JC13 Rec'd PCT/PTO 19 FEB 2002

FORM PTO 1390
(REV 5-93) US DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. §371

ATTORNEY DOCKET NUMBER
2002_0256A

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)
NEW 10/049822

International Application No.	International Filing Date	Priority Date Claimed
PCT/JP00/05502	August 17, 2000	August 17, 1999

Title of Invention
A GENETICALLY ENGINEERED cDNA OF RAT bcl-x GENE AND AN IMPROVED PROTEIN

Applicant(s) For DO/EO/US
Shigeo OHTA, Sadamitsu ASOH

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. §371.

2. [] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. §371.

3. [] This express request to begin national examination procedures (35 U.S.C. §371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. §371(b) and PCT Articles 22 and 39(1).

4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. [X] A copy of the International Application as filed (35 U.S.C. §371(c)(2))
   a. [] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] has been transmitted by the International Bureau.
   c. [] is not required, as the application was filed in the United States Receiving Office (RO/US)

6. [X] A translation of the International Application into English (35 U.S.C. §371(c)(2)). **ATTACHMENT A**

7. [] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. §371(c)(3)).
   a. [] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [] have been transmitted by the International Bureau.
   c. [] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [] have not been made and will not be made.

8. [] A translation of the amendments to the claims under PCT Article 19.

9. [X] An unexecuted oath or declaration of the inventor(s) (35 U.S.C. §371(c)(4)). **ATTACHMENT B**

10. [] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. §371(c)(5)).

Items 11. to 14. below concern other document(s) or information included:

11. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98. **ATTACHMENT C**

12. [] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. [X] A FIRST preliminary amendment. **ATTACHMENT D**

    [] A SECOND or SUBSEQUENT preliminary amendment.

14. [] Other items or information:

THE COMMISSIONER IS AUTHORIZED TO CHARGE ANY DEFICIENCY IN THE FEE FOR THIS PAPER TO DEPOSIT ACCOUNT NO. 23-0975.

U.S. APPLICATION NO. (if known, see 37 CFR 1.5) NEW 10/049822	INTERNATIONAL APPLICATION NO. PCT/JP00/05502	ATTORNEY'S DOCKET NO. 2002_0256A

15. [X] The following fees are submitted

	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):		
Neither international preliminary examination fee nor international search fee paid to USPTO and International Search Report not prepared by the EPO or JPO $1040.00		
International Search Report has been prepared by the EPO or JPO . . . . . $ 890.00		
International preliminary examination fee not paid to USPTO but international search paid to USPTO . . . . . $ 740.00		
International preliminary examination fee paid to USPTO but claims did not satisfy provisions of PCT Article 33(1)-(4) . . . . . $ 690.00		
International preliminary examination fee paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) . . . . . $ 100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$890.00	
Surcharge of $130.00 for furnishing the oath or declaration later than [] 20 [] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	-20 =		X $18.00	$	
Independent Claims	- 3 =		X $84.00	$	
Multiple dependent claim(s) (if applicable)			+ $280.00	$	
TOTAL OF ABOVE CALCULATIONS =				$890.00	
[] Small Entity Status is hereby asserted. Above fees are reduced by 1/2.				$	
SUBTOTAL =				$890.00	
Processing fee of $130.00 for furnishing the English translation later than [] 20 [] 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$890.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40 per property +				$	
TOTAL FEES ENCLOSED =				$890.00	
				Amount to be refunded	$
				Amount to be charged	$

a. [X] A check in the amount of $890.00 to cover the above fees is enclosed. A duplicate copy of this form is enclosed.

b. [] Please charge my Deposit Account No. 23-0975 in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 23-0975.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

19. CORRESPONDENCE ADDRESS

000513

PATENT TRADEMARK OFFICE

By: Warren Cheek

Warren M. Cheek, Jr.,
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February 19, 2002

[CHECK NO. 48934]

[2002_0256A]

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents, Box PCT
United States Patent and Trademark Office
Washington, D.C. 20231
www.uspto.gov

U S APPLICATION NUMBER NO.	FIRST NAMED APPLICANT	ATTY. DOCKET NO
10/049,822	Shigio Ohta	2002_0256A

INTERNATIONAL APPLICATION NO.	
PCT/JP00/05502	
I A. FILING DATE	PRIORITY DATE
08/17/2000	08/17/1999

000513
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2033 K STREET N. W.
SUITE 800
WASHINGTON, DC 20006-1021

CONFIRMATION NO. 6325
371 FORMALITIES LETTER

OC000000008194244

Date Mailed: 05/30/2002

NOTIFICATION OF DEFECTIVE RESPONSE

The following items have been submitted by the applicant or the IB to the United States Patent and Trademark Office as an Elected Office (37 CFR 1.495):

- U.S. Basic National Fee
- Priority Document
- Biochemical Sequence Listing
- Copy of IPE Report
- Copy of references cited in ISR
- Copy of the International Application
- Copy of the International Search Report
- Information Disclosure Statements
- Oath or Declaration
- Preliminary Amendments

RECEIVED
JUN - 3 2002
WENDEROTH, LIND & PONACK

The following items **MUST** be furnished within the period set forth below in order to complete the requirements for acceptance under 35 U.S.C. 371:

Applicant is required to complete the response within a time limit of ONE MONTH from the date of this Notification or within the time remaining in the response set forth in the Notification of Missing Requirements, whichever is the longer. No extension of this time limit may be granted under 37 CFR 1.136, but the period for response set in the Notification of Missing Requirements may be extended under 37 CFR 1.136(a).

The following items **MUST** be furnished within the period set forth below:

- The nucleotide and/or amino acid sequence disclosure contained in this application does not comply with the requirements for such a disclosure as set forth in 37 CFR 1.821-1.825 for the following reason(s):

  - A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 CFR 1.821(e).
  - APPLICANT MUST PROVIDE

- An initial or substitute computer readable form (CRF) of the "Sequence Listing."

- For questions regarding compliance to 37 CFR 1.821-1.825 requirements, please contact:
  - For Rules Interpretation, call (703) 308-4216
  - To Purchase PatentIn Software, call (703) 306-2600
  - For PatentIn Software Program Help, call (703) 306-4119 or e-mail at patin21help@uspto.gov or patin3help@uspto.gov

- A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 CFR 1.821(e).

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

*A copy of this notice **MUST** be returned with the response.*

INDIA L EVANS

Telephone: (703) 305-2936

PART 1 - ATTORNEY/APPLICANT COPY

U.S. APPLICATION NUMBER NO	INTERNATIONAL APPLICATION NO	ATTY DOCKET NO.
10/049,822	PCT/JP00/05502	2002_0256A

FORM PCT/DO/EO/916 (371 Formalities Notice)

10/049822
JC13 Rec'd PCT/PTO 19 FEB 2002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of :

Shigeo OHTA et al. : **Attn: BOX PCT**

Serial No. NEW : **Docket No. 2002_0256A**

Filed February 19, 2002 :

A GENETICALLY ENGINEERED cDNA OF
RAT bcl-x GENE AND AN IMPROVED PROTEIN
[Corresponding to PCT/JP00/05502
Filed August 17, 2000]

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents,
Washington, DC 20231

Sir:

Prior to calculating the filing fee, please amend the above-identified application as follows:

IN THE SPECIFICATION

Page 1, immediately after the title, please insert:

This application is a 371 of PCT/JP00/05502 filed August 17, 2000.

Page 6, lines 17-18, please replace with the following sentence:

Fig. 21 is a microscopic photograph of the chondrocytes in cartilage slice incubated in a culture medium containing the protein TAT-Bcl-x_L for 5 days.

Page 6, lines 20-21, please replace with the following sentence:

Fig. 22 is a microscopic photograph of the chondrocytes in cartilage slice incubated in a culture medium containing the protein TAT-Bcl-x_L for 9 days.

ATTACHMENT D

Page 23, lines 3-5, please replace with the following sentence:

· Reaction solution (volume 100 μl): 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM each dATP, dCTP, dTTP, dGTP, AmpliTaqGOLD: 2.5U

Page 23, line 20 to page 24, line 2, please replace with the following paragraph as follows:

After the reaction, the PCR solution (75 μl) containing Primer 12 (SEQ ID NO: 17), Primer 10 (final concentration: 1 μM each), and AmpliTaqGOLD (2.5 U) was added, and the PCR was carried out according to the above reaction condition 3. Primer 12 is a sense sequence coding for Met-Gly and the subsequent N-terminal three amino acid residues of TAT-PTD with a cleavage site of the restriction enzyme *Nde* I at the 5'end. The amplified DNA fragment was purified by polyacrylamide gel electrophoresis. After cleavage with *Nde* I, the cleaved end was made blunt with T4DNA polymerase and further subjected to be digested with *Hind* III. The *Escherichia coli* expression vector pROEX1 (Life Technologies) was cleaved with *Nco* I, then made blunt with nuclease S1, followed by digestion with *Hind* III. Two DNAs were ligated each other to yield the recombinant vector pROEX1- *bcl-x*Y22F/Q26N/R165K coding for TAT-Bcl-xFNK in which a protein-transduction-domain peptide of TAT protein is fused at the N-terminal.

IN THE CLAIMS

Please amend the claims as follows:

4. **(Amended)** A recombinant vector carrying the genetically engineered cDNA of claim 1.

Please add the following new claims:

9. **(New)** A recombinant vector carrying the genetically engineered cDNA of claim 2.

10. **(New)** A recombinant vector carrying the genetically engineered cDNA of claim 3.

11. **(New)** A cell into which the recombinant vector of claim 9 was introduced.

12. **(New)** A cell into which the recombinant vector of claim 10 was introduced.

REMARKS

The specification has been amended to reflect the national stage status. In addition, the claims have been amended to remove the multiple dependencies to reduce the PTO filing fee.

The specification has also been amended to correct certain typographical errors. The amendments to page 6 are supported by Example 11 beginning on page 26 of the specification. The amendments to page 23 are to correct obvious errors.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached pages are captioned **"Version with markings to show changes made."**

Favorable action on the merits is solicited.

Respectfully submitted,

Shigeo OHTA et al.

By _____
Warren M. Cheek, Jr.
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February 19, 2002

OIPE JC98 JUN 25 2002 PATENT & TRADEMARK OFFICE

DT15 Rec'd PCT/PTO JUN 25 2002

N/A #7

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	:	**Confirmation No. 6325**
Shigeo OHTA et al.	:	Docket No. 2002-0256A
Serial No. 10/049,822	:	Group Art Unit Not Yet Assigned
Filed April 1, 2002	:	Examiner Not Yet Assigned
A GENETICALLY ENGINEERED cDNA OF RAT bcl-x GENE AND AN IMPROVED PROTEIN	:	THE COMMISSIONER IS AUTHORIZED TO CHARGE ANY DEFICIENCY IN THE FEES FOR THIS PAPER TO DEPOSIT ACCOUNT NO. 23-0975

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents,
Washington, D.C. 20231

Sir:

Responsive to the Notice dated May 30, 2002, please amend the above-identified application as follows:

In the Specification:

Page 1, line 1, delete in its entirety.

between lines 3 and 6, insert the following new heading:

Background of the Invention

line 6, replace the heading with the following new heading:

1. Field of the Invention

line 17, replace the heading with the following new heading:

2. Description of the Related Art

Page 3, line 10, replace the heading with the following new heading:

Summary of the Invention

Page 4, replace the paragraph beginning at line 3 with the following paragraph:

(6) An improved protein produced from the genetically engineered cDNA of said invention (1), which has at least one amino acid substitution in SEQ ID NO: 2, which the amino acid substitution is selected from the substitutions of residues 22 Tyr with Phe, residues 26 Gln with Asn and residues 165 Arg with Lys.

line 16, replace the heading with the following new heading:

Brief Description of the Drawings

Page 5, replace the paragraph beginning at line 20 with the following paragraph:

Fig. 12 shows the results of a test for resistance of the transfected cells to heat treatment.

replace the paragraph beginning at line 26 with the following paragraph:

Fig. 14 shows the results of a test for dehydrogenase activity in the transfected cells treated with TN-16 by the WST-1 assay.

replace the paragraph beginning at line 29 with the following paragraph:

Fig. 15 shows the results of a test for dehydrogenase activity in the transfected cells treated with staurosporine by the WST-1 assay.

Page 7, line 11, replace the heading with the following new heading:

Description of the Preferred Embodiments

replace the paragraph beginning at line 13 with the following paragraph:

The engineered cDNA of the invention as indicated in the above invention (1), is characterized by having at least one substitution selected from the substitutions that change the codon of residues 22 Tyr (tac) to the codons of Phe (ttt/ttc), the codon of residues 26 Gln (cag) to the codons of Asn (aat/aac) and the codon of residues 165 Arg (cgg) to the codons of Lys (aaa/aag), in the coding region of rat *bcl-x* cDNA of SEQ ID NO: 1. In a preferred embodiment, the nucleotide substitutions have occurred in all the above 3 sites in the engineered cDNA of the invention (1). The engineered cDNA which has the nucleotide substitutions at the 3 sites produces the improved protein Bcl-xFNK as shown in the amino acid sequence of SEQ ID NO: 3. In this improved protein Bcl-xFNK, three hydrogen bonds between Tyr^{22} and Asp^{156}, between Gln^{26} and Ser^{164} and between Arg^{165} and Pro^{116}, which are formed in the wild-type rat Bcl-x_L as shown in the three-dimensional structure of Fig. 1, are disturbed as a result of the amino acid substitutions (Tyr22Phe; Gln26Asn; Arg165Lys) caused by the above-described nucleotide substitutions.

Page 9, replace the paragraph beginning at line 9 with the following paragraph:

Among the cells of the invention (5), particularly, the mammalian cells can be also proliferated in a serum-free medium as shown in the data of the Examples below. In general, in order to keep a cultured cell alive for a certain period of time, it is necessary to add a serum (e.g., fetal bovine serum) containing growth factors to the culture medium. Addition of the growth factors can inhibit apoptosis of the cells to prolong the cellular life span. When cellular products such as physiologically active substances or monoclonal antibodies are recovered and purified from the culture medium in which mammalian cells are grown, however, it is desirous that the culture medium contains no impurities such as serum. The reason is that the cost increases and

extra steps are required for purifying the objective substance, and that there is a possibility of the serum containing a risk factor such as a virus. The use of the serum-free and protein-free medium containing no serum, however, practically reduces the degree of cell growth and results in increasing dead cells, when the serum-free and protein-free media have been used. In addition, there is a problem that such increased dead cells might cause contamination of the cellular contents into the culture medium because of an outflow of the cellular contents from the dead cells.

Page 14, replace the paragraph beginning at line 5 with the following paragraph:

To construct *bcl-x*Y22F/Q26N, Gln^{26} was first substituted with Asn, followed by the amino acid substitution of Tyr^{22} with Phe. PCR was carried out using pEF1-BOS*bcl-x* (50 ng) as a template and a pair of the above Primer 1 and Primer 5 (SEQ ID NO:8). Another PCR was carried out using pEF1-BOS*bcl-x* (50 ng) as a template and a pair of the above Primer 4 and Primer 6 (SEQ ID NO:9). The components of the reaction solution (100ml) were the same as above, and the reactions were performed according to the above-described condition 1. Primer 5 is the antisense sequence of *bcl-x*cDNA and contains the nucleotide substitutions to convert the codon of Gln^{26} into a codon coding for Asn. Primer 6 is the sense sequence of *bcl-x* cDNA and contains the nucleotide substitutions to convert the codon of Gln^{26} into a codon coding for Asn. The nucleotide sequence of 5'-end half of Primer 6 is complementary to that of 5'-end half of Primer 5. Two PCR products amplified by PCR were purified by polyacrylamide gel electrophoresis, and two DNA fragments (6 ng each) were mixed to synthesize the respective complementary strand using AmpliTaqGOLD. The condition for synthesis was the same as the

above reaction condition 2. After the reaction, a PCR reaction solution (75ml) containing Primer 1, Primer 4 (final concentration: 1 μM each) and AmpliTaqGOLD (2.5U) were added, and the PCR was carried out according to the above reaction condition 1. The 650-bp PCR product was purified by polyacrylamide gel electrophoresis and then treated with the restriction enzyme *Bam*H I. On the other hand, pEF1-BOSbcl-x was treated with *Bam*H I to yield two DNA fragments (5650 bp and 650 bp). The longer DNA fragment (5650 bp) was ligated to the above PCR product in a right orientation to yield the clone pEF1-BOS*bcl-x*Q26N having an amino substitution of Gln26Asn.

Page 16, replace the paragraph beginning at line 15 with the following paragraph:

The cells (FDC-P1 or Jurkat) were washed 3 times with an ice-cold K-PBS solution (30.8 mM NaCl, 120.7 mM KCl, 8.1 mM Na_2HPO_4, 1.46mM KH_2PO_4) and suspended in K-PBS containing 5 mM $MgCl_2$ (Mg-K-PBS) at 10^7 cells/ml. The cell suspension (0.4 ml) was mixed with Mg-K-PBS solution (0.4 ml) in an ice-cold cuvette (Electroporation Cuvettes Plus, 4-mm Gap, BTX, A Division of Genetronics). Then, the linearized pEF1-BOS*bcl-x*Y22F/Q26N/R165K (10 μg) and the linearized DNA pST-neoB (0.5 μg) containing a drug geneticin-resistant gene were added thereto. Change of the volume by addition of the DNA was kept up to 1%. After 10-min incubation on ice, electroporation was carried out to introduce the recombinant vector into cells using the Gene Pulser (250 μF and 330V, BioRad). After 10-min incubation on ice, the cells were gently suspended in 39 ml of the fresh culture medium in a 100-mm dish and incubated in a CO_2 incubator. After a lapse of 1 day, the cells were divided and placed in a 96-well plate. Geneticin (GIBCO BRL) was added at 200 μg/ml for FDC-P1 cells and

at 1 mg/ml for Jurkat cells to select the geneticin-resistant cells.

Page 17, replace the paragraph beginning at line 25 with the following paragraph:

The results are shown in Fig. 2. It was confirmed that the cell transfected with the recombinant vector pEF1-BOS*bcl-x*FNK expresses a protein having the same molecular weight (about 30 kDa) as that expressed in the cells transfected with the clone pEF1-BOS*bcl-x* of the wild-type Bcl-x_L.

Page 20, replace the paragraph beginning at line 21 with the following paragraph:

The results are shown in Figs. 14 and 15. It was confirmed that all independent transfectants expressing the improved Bcl-xFNK exhibited high resistance to treatment with TN-16 and staurosporine, and the dehydrogenase activity was maintained at high level.

Page 22, replace the paragraph beginning at line 23 with the following paragraph:

The engineered cDNA coding for Bcl-xFNK prepared in Example 1 was fused to the cDNA coding for the protein transduction domain of TAT protein of HIV virus by 2-step PCR. PCR was carried out using Primer 9 (SEQ ID NO: 14) as the 5'-end primer, Primer 10 (SEQ ID NO: 15) as the 3'-end primer and the recombinant vector pEF1-BOS*bcl-x*Y22F/Q26N/R165K having Bcl-xFNK cDNA as a template. Primer 9 is the sense sequence consisting of the 3'-end of TAT-PTD cDNA and the 5'-end (containing the initiation codon) of Bcl-xFNK cDNA. Primer 10 is the antisense sequence consisting of the 3'-end (containing a termination codon) of Bcl-xFNK cDNA and the cleavage site for the restriction enzyme *Hin*d III. Details of the PCR reaction are as follows

- Reaction solution (volume 100 μl): 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM

$MgCl_2$, 0.001% gelatin, 0.2 mM each dATP, dCTP, dTTP, dGTP, , AmpliTaqGOLD: 2.5U

- Primers: a combination of Primer 9 and Primer 10 (each primer: 1 μM)
- Template DNA: 50 ng
- Reaction condition 3: 94°C/10 min (94°C/30 sec; 49°C/30 sec; 72°C/1 min) ×15

cycles.

Page 23, replace the paragraph beginning at line 20 with the following paragraph:

After the reaction, the PCR solution (75 μl) containing Primer 12 (SEQ ID NO: 17), Primer 10 (final concentration: 1 μM each), and AmpliTaqGOLD (2.5 U) was added, and the PCR was carried out according to the above reaction condition 3. Primer 12 is a sense sequence coding for Met-Gly and the subsequent N-terminal three amino acid residues of TAT-PTD with a cleavage site of the restriction enzyme *Nde* I at the 5'-end. The amplified DNA fragment was purified by polyacrylamide gel electrophoresis. After cleavage with *Nde* I, the cleaved end was made blunt with T4DNA polymerase and further subjected to be digested with *Hin*d III. The *Escherichia coli* expression vector pROEX1 (Life Technologies) was cleaved with *Nco* I, then made blunt with nuclease S1, followed by digestion with Hind III. Two DNAs were ligated to each other to yield the recombinant vector pROEX1- -*bcl-x*Y22F/Q26N/R165K coding for TAT-Bcl-xFNK in which a protein-transduction-domain peptide of TAT protein is fused at the N-terminal.

Page 27, line 29 delete the entire heading.

In the Abstract:

Page 30, line 1, replace the heading with the following new heading:

ABSTRACT OF THE DISCLOSURE

In the Sequence Listing:

Please replace the Sequence Listing of record with the attached substitute Sequence Listing.

In the Claims:

Above claim 1, insert the following:

What is claimed is:

Please add the following new claims.

13. An isolated polynucleotide comprising the nucleotide sequence of SEQ ID NO: 1, said SEQ ID NO: 1 having at least one nucleotide substitution which changes, in the amino acid sequence encoded by SEQ ID NO: 1, Tyr at amino acid residue 22 to Phe, Gln at amino acid residue 26 to Asn, Arg at amino acid residue 165 to Lys, or a combination thereof.

14. The polynucleotide of claim 13, which is attached at its 5'-end with an oligonucleotide encoding a protein-transduction-domain peptide.

15. The polynucleotide of claim 14, wherein the oligonucleotide encodes the amino acid sequence of SEQ ID NO: 12 or 13.

16. A recombinant vector comprising the polynucleotide of claim 13.

17. A cell into which the recombinant vector of claim 16 was introduced.

18. An isolated polypeptide comprising the amino acid sequence of SEQ ID NO: 2, said SEQ ID NO: 2 having at least one amino acid substitution selected from the group consisting of substitution of Tyr at amino acid residue 22 to Phe, substitution of Gln at amino acid residue 26 to Asn and substitution of Arg at amino acid residue 165 to Lys.

19. The polypeptide of claim 18, which is attached at the N-terminal with a protein-transduction-domain peptide.

20. The polypeptide of claim 19, wherein the protein-transduction-domain peptide is an oligopeptide comprising the amino acid sequence of SEQ ID NO: 12 or 13.

21. A recombinant vector comprising the polynucleotide of claim 14.

22. A recombinant vector comprising the polynucleotide of claim 15.

23. A cell into which the recombinant vector of claim 21 was introduced.

24. A cell into which the recombinant vector of claim 22 was introduced.

25. The polypeptide of claim 18, wherein said SEQ ID NO: 2 has an amino acid substitution selected from the group consisting of substitution of Tyr at amino acid residue 22 to Phe, substitution of Gln at amino acid residue 26 to Asn, substitution of Arg at amino acid residue 165 to Lys and a combination thereof.

REMARKS

The foregoing amendments are presented to place the application in better form and in compliance with the sequence rules under 37 CFR 1.821-1.825. In particular, the specification headings have been amended in conformance with U. S. practice, and minor grammatical and spelling errors have been corrected.

Applicants have also submitted a Sequence Listing in both paper and computer readable form as required by 37 C.F.R. 1.821(c) and (e). Amendments directing its entry into the specification have also been incorporated herein. The content of the paper and computer readable copies are the same and no new matter has been added.

Applicants have also added new claims 13-25 to further protect the present invention. Support for the new claims is readily apparent from the teachings of the specification and the original claims.

Attached hereto is a marked-up version of the changes made to the specification by the current amendment. The attached page is captioned "**Version with markings to show changes made.**"

In view of the foregoing, it is believed that each requirement set forth in the Notice has been satisfied, and that the application is now in compliance with the sequence rules under 37 CFR 1.821-1.825. Accordingly, favorable examination on the merits is respectfully requested.

Respectfully submitted,

Shigeo OHTA et al.

By: _____
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June 25, 2002

Version with Markings to Show Changes Made

~~DESCRIPTION~~

A Genetically Engineered cDNA of Rat *bcl-x* Gene and An Improved Protein

Background of the Invention

1.

~~Technical~~ Field of the Invention

The present invention relates to a genetically engineered cDNA of rat *bcl-x* gene and an improved protein. More particularly, it relates to a novel cDNA expressing an improved protein of Bcl-x_L having higher apoptosis-inhibiting activity and cell death-inhibiting activity than the protein Bcl-x_L expressed by the rat's apoptosis-inhibiting gene *bcl-x*. The invention also relates to materials for utilizing such cDNA in gene engineering, as well as to an improved protein of Bcl-x_L expressed by the cDNA.

2. Description of the Related

~~Background~~ Art

Apoptosis is one of programmed cell death. Apoptosis is accompanied by poor contact with the surrounding cells, concentration of cytoplasm, condensation of the chromatin and nuclei associated with the endonuclease activity, fragmentation of the nuclei, formation of membrane-bounded apoptotic bodies, and phagocytosis of the apoptotic bodies by the adjacent macrophage or epithelial cells. A phenomenon that the chromosomal DNA is cleaved into DNA fragments of 180 to 200 base length by the endonuclease activity is also observed. Such phenomena have been discussed as the mechanism indicating that the apoptotic bodies are finally phagocytosed by the adjacent cells (for example, Immunology Today 7:115-119, 1986; Science 245:301-305, 1989).

As a gene controlling the apoptosis, for example, the gene *bcl-2* which is

Bcl-x_L in cells.

Another purpose of the invention is to provide a recombinant vector containing this engineered cDNA and a cell having the recombinant vector.

Still another purpose of the invention is to provide the improved protein expressed from the above-described engineered cDNA.

Summary the Disclosure of Invention

In order to solve the above-described problems, the present application provides the following inventions (1) to (8).

(1) A genetically engineered cDNA of the rat *bcl-x* gene, which has at least one substitution selected from the substitutions that change residues 22 Tyr to Phe, residues 26 Gln to Asn and residues 165 Arg to Lys, in the coding region of rat *bcl-x* cDNA of SEQ ID NO: 1.

(2) The genetically engineered cDNA of said invention (1), which is attached at its 5'-end with an oligonucleotide encoding a protein-transduction-domain peptide.

(3) The genetically engineered cDNA of said invention (2), wherein the oligonucleotide encodes the amino acid sequence of SEQ ID NO: 12 or 13.

(4) A recombinant vector carrying the genetically engineered cDNA of any one of said inventions (1) to (3).

(5) A cell into which the recombinant vector of said invention (4) is

introduced.

(6) An improved protein produced from the genetically engineered cDNA of said invention (1), which has at least one amino acid substitution in SEQ ID NO: 2, which the amino acid substitution is selected from the substitutions of residues 22 Tyr with Phe, ~~reisdues~~ residues 26 Gln with Asn and residues 165 Arg with Lys.

(7). The improved protein of said invention (6), which is attached at the N-terminal with a protein-transduction-domain peptide.

(8). The improved protein of said invention (7), wherein the protein-transduction-domain peptide is an oligopeptide having the amino acid sequence of SEQ ID NO: 12 or 13.

Brief Description of the Drawings

Fig. 1 shows the three-dimensional structure of the wild-type rat Bcl-x_L.

Fig. 2 shows the results of Western blotting analysis of the expression level of the improved protein Bcl-xLFNK in the transfected cell.

Fig. 3 shows the results of a test for resistance of the transfected cells to apoptosis induced by serum depletion.

Fig. 4 shows the results of a test for resistance of the transfected cells to anti-Fas antibody.

Fig. 5 shows the results of a test for resistance of the transfected cells to staurosporine.

Fig. 6 shows the results of a test for resistance of the transfected cells to TN-16.

Fig. 7 shows the results of a test for resistance of the transfected cells to camptothecin.

Fig. 8 shows the results of a test for resistance of the transfected cells to hydroxyurea.

Fig. 9 shows the results of a test for resistance of the transfected cells to trichostatin A.

Fig. 10 shows the results of a test for resistance of the transfected cells to hydrogen peroxide.

Fig. 11 shows the results of a test for resistance of the transfected cells to paraquat.

Fig. 12 shows the results of a test for resistance of the transfected cells to heat treatment.

Fig. 13 shows the results of a test for dehydrogenase activity in the transfected cells after heat treatment by the WST-1 assay.

Fig. 14 shows the results of a test for dehydrogenase activity in the transfected cells ~~treatmented~~ treated with TN-16 by the WST-1 assay.

Fig. 15 shows the results of a test for dehydrogenase activity in the transfected cells ~~treatmented~~ treated with staurosporine by the WST-1 assay.

administration of dexamethasone.

Fig. 26 is a microscopic photograph showing the liver slice of a mouse to which a solvent (PBS) has been administered systemically, followed by administration of dexamethasone.

Fig. 27 is a microscopic photograph showing the liver slice of a mouse to which a solvent (PBS) only has been administered systemically.

Description of the Preferred Embodiments

~~The Best Mode for Carrying Out the Invention~~

The engineered cDNA of the invention as indicated in the above invention (1), is characterized by having at least one substitution selected from the substitutions that change the codon of residues 22 Tyr (tac) to the codons of Phe (ttt/ttc), the codon of residues 26 Gln (cag) to the codons of Asn (aat/aac) and the codon of residues 165 Arg (cgg) to the codons of Lys (aaa/aag), in the coding region of rat *bcl-x* cDNA of SEQ ID NO: 1. In a preferred embodiment, the nucleotide substitutions have occurred in all the above 3 sites in the engineered cDNA of the invention (1). The engineered cDNA which has the nucleotide substitutions of at the 3 sites produces the improved protein Bcl-xFNK as shown in the amino acid sequence of SEQ ID NO: 3. In this improved protein Bcl-xFNK, three hydrogen bonds between Tyr^{22} and Asp^{156}, between Gln^{26} and Ser^{164} and between Arg^{165} and Pro^{116}, which are formed in the wild-type rat Bcl-x_L as shown in the three-dimensional structure of Fig. 1, are disturbed as a result of the amino acid substitutions (Tyr22Phe; Gln26Asn; Arg165Lys) caused by the above-described nucleotide substitutions.

The engineered cDNA may be prepared by a known method with a mutation kit or a PCR method as mentioned in Examples using the rat *bcl-x* cDNA

invention (4) can be introduced into all of the cells, for example, prokaryotic cells such as *Escherichia coli* or *Bacillus subtilis*; eukaryotic cells such as yeast, insect cells, mammalian cells, or plant cells are included. Introduction of the recombinant vector into cells may be achieved by a known method. For example, when the recombinant vector is introduced into a mammalian cell, electroporation, a calcium phosphate method, a liposome method, a DEAE dextran method, and the like may be employed.

Among the cells of the invention (5), particularly, the mammalian cells can be also proliferated in a serum-free medium as shown in the data of the Examples below. In general, in order to keep a cultured cell alive for a certain period of time, it is necessary to add a serum (e.g., fetal bovine serum) containing growth factors to the culture medium. Addition of the growth factors can inhibit apoptosis of the cells to prolong the cellular life span. When cellular products such as physiologically active substances or monoclonal antibodies are recovered and purified from the culture medium in which mammalian cells are grown, however, it is desirous that the culture medium contains no impurities such as serum. The reason is that the cost increases and extra steps are required for purifying the objective substance, and that there is a possibility of the serum containing a risk factor such as a virus. The use of the serum-free and protein-free medium containing no serum, however, practically reduces the degree of cell growth and results in increasing dead cells, when the serum-free and protein-free media have been used. In addition, there is a problem that such increased dead cells might cause contamination of the ~~cellular~~ cellular contents into the culture medium because of an outflow of the cellular contents from the dead cells.

On the other hand, there is an alternative method for proliferating cells without using any growth factor, wherein the cells are transfected with an proto-oncogene. In this method, however, it has been elucidated that apoptosis is rather promoted by expression of multiple proto-oncogene products.

(having two *Bam*H I sites) was treated with *Bam*H I to yield two DNA fragments (5650 bp and 650 bp). The longer DNA fragment (5650 bp) was ligated to the above PCR product in a right orientation to yield the clone pEF1-BOS*bcl-x*R165K having an amino acid substitution of Arg165Lys.

To construct *bcl-x*Y22F/Q26N, Gln^{26} was first substituted with Asn, followed by the amino acid substitution of Tyr^{22} with Phe. PCR was carried out using pEF1-BOS*bcl-x* (50 ng) as a template and a pair of the above Primer 1 and Primer 5 (SEQ ID NO:8). Another PCR was carried out using pEF1-BOS*bcl-x* (50 ng) as a template and a pair of the above Primer 4 and Primer 6 (SEQ ID NO:9). The components of the reaction solution (100µl) were the same as above, and the reactions were performed according to the above-described condition 1. Primer 5 is the antisense sequence of *bcl-x*cDNA and contains the nucleotide substitutions to convert the codon of Gln^{26} into a codon coding for Asn. Primer 6 is the sense sequence of *bcl-x* cDNA and contains the nucleotide substitutions to convert the codon of Gln^{26} into a codon coding for Asn. The ~~nucelotide~~ nucleotide sequence of 5'-end half of Primer 6 is complementary to that of 5'-end half of Primer 5. Two PCR products amplified by PCR were purified by polyacrylamide gel electrophoresis, and two DNA fragments (6 ng each) were mixed to synthesize the respective complementary strand using AmpliTaqGOLD. The condition for synthesis was the same as the above reaction condition 2. After the reaction, a PCR reaction solution (75µl) containing Primer 1, Primer 4 (final concentration: 1 µM each) and AmpliTaqGOLD (2.5U) were added, and the PCR was carried out according to the above reaction condition 1. The 650-bp PCR product was purified by polyacrylamide gel electrophoresis and then treated with the restriction enzyme *Bam*H I. On the other hand, pEF1-BOSbcl-x was treated with *Bam*H I to yield two DNA fragments (5650 bp and 650 bp). The longer DNA fragment (5650 bp) was ligated to the above PCR product in a right orientation to yield the clone pEF1-BOS*bcl-x*Q26N having an amino substitution of Gln26Asn.

Then, two PCR reactions were carried out independently using pEF1-BOS*bcl-x*Q26N as a template. One PCR contained a pair of the above Primer

Example 2

Preparation of Transfected Cells

A murine premyeloid cell line FDC-P1 were cultured on RPMI1640 medium containing fetal bovine serum (10%) and a cytokine IL-3 (the supernatant of WEHI cell culture broth). A human leukemia cell line Jurkat were cultured on RPMI1640 medium containing fetal bovine serum (10%). The cells were incubated in a CO_2 incubator (5% CO_2/95% air, 37°C).

The recombinant vector pEF1-BOS*bcl-x*Y22F/Q26N/R165K prepared in Example 1 was amplified in *Escherichia coli* DH5αMCR (GIBCO BRL) and prepared using the Qiagen Plasmid midi Kit (Qiagen). The recombinant vector was cleaved with *Sca* I (one cleavage site) and the resulting linear DNA was dissolved in 1 mM EDTA solution.

The cells (FDC-P1 or Jurkat) were washed 3 times with an ice-cold K-PBS solution (30.8 mM NaCl, 120.7 mM KCl, 8.1 mM Na_2HPO_4, 1.46mM KH_2PO_4) and suspended in K-PBS ~~containig~~ containing 5 mM $MgCl_2$ (Mg-K-PBS) at 10^7 cells/ml. The cell suspension (0.4 ml) was mixed with Mg-K-PBS solution (0.4 ml) in an ice-cold cuvette (Electroporation Cuvettes Plus, 4-mm Gap, BTX, A Division of Genetronics). Then, the linearized pEF1-BOS*bcl-x*Y22F/Q26N/R165K (10 μg) and the linearized DNA pST-neoB (0.5 μg) containing a drug geneticin-resistant gene were added thereto. Change of the volume by addition of the DNA was kept up to 1%. After 10-min incubation on ice, electroporation was carried out to introduce the recombinant vector into cells using the Gene Pulser (250 μF and 330V, BioRad). After 10-min incubation on ice, the cells were gently suspended in 39 ml of the fresh culture medium in a 100-mm dish and incubated in a CO_2 incubator. After a lapse of 1 day, the cells were divided and placed in a 96-well plate. Geneticin (GIBCO BRL) was added at 200 μg/ml for FDC-P1 cells and at 1 mg/ml for Jurkat cells to select the geneticin-resistant cells.

Example 3

Analysis of expression level of the improved Bcl-xFNK

The expression level of the improved protein Bcl-xFNK in the transfected cells prepared in Example 2 was examined by Western blotting. The cells were washed once with PBS (pH 7.4; NaCl 137 mM, Na_2HPO_4 8.1 mM, KCl 2.68 mM, KH_2PO_4 1.47 mM). After addition of 2% SDS (sodium dodecylsulfate) solution, the cells were disrupted by sonication to solubilize the whole proteins. The proteins were quantitatively analyzed by the BCA Protein Assay (PIERCE), and 20 μg of protein was fractionated on SDS-polyacrylamide gel electrophoresis (Lemli's system). After the electrophoresis, the protein was blotted on a PVDF membrane (Amersham Pharmacia Biotec). The membrane was immersed in a blocking solution containing fetal bovine serum (10%), and then immersed in a TBS solution (Tris-HCl pH 7.4 20 mM, NaCl 136 mM, Tween 80 0.2%) containing the murine monoclonal antibody 105-1 (0.5 μg/ml) that reacts with the C-terminal of rat Bcl-x_L. After incubation at 37°C for 1 hour, the membrane was washed well with TBS, then immersed in a TBS solution containing an HRP (horse radish peroxidase)-binding or AP (alkaline phosphatase)-conjugated secondary antibody, and incubated at 37°C for 1 hour. The HRP-conjugated secondary antibodies binding to Bcl-x_L and Bcl-xFNK were visualized on an X-ray film using the RENAISSANCE kit (NEN Life Science Product). The AP-conjugated antibodies binding to Bcl-x_L and Bcl-xFNK were visualized with the fluoro-image analyzer FLA-2000 (Fuji film) using the ATTOPHOS kit (Boehringer).

The results are shown in Fig. 2. It was confirmed that the cell transfected with the recombinant vector pEF1-BOS*bcl-x*FNK expresses a protein having the same molecular weight (about 30 kDa) as that expressed in the cells ~~transfeetd~~ transfected with the clone pEF1-BOS*bcl-x* of the wild-type Bcl-x_L.

Example 5

Confirmation of the resistance to the cell death of the FDC-P1*bcl-x*FNK transfectants

For the transfectant FDC-P1*bcl-x*FNK cells prepared in Example 2, the resistance was examined to a variety of apoptosis-inducing stimuli. The results are shown in Figs. 14 to 16. In these figures, the empty marks, ◇, □, △, ▽, and ○ represent 5 independent transfectants, FDC-P1*bcl-x*FNK -1, -2, -3, -4, and -5, respectively. The mark ● represents the transfectant FDC-P1*bcl-x* expressing the wild-type Bcl-x_L at the same expression level. The mark ■ represents FDC-P1*vec* in which the empty vector plasmid DNA has been introduced.

(a) Resistance to TN-16 and staurosporine

The cells were suspended in culture medium at 2×10^5 cells/ml, to which TN-16 (50 μM) and staurosporine (10 nM) was then added. The dehydrogenase activity of the cells (100 μl of the culture broth) was determined daily using the Cell Counting Kit (Dojin Chemical) and WST-1 as a substrate (WST-1 Assay). The enzyme activity was taken as 100% immediately before addition of the agents.

The results are shown in Figs. 14 and 15. It was confirmed that all independent transfectants expressing the improved Bcl-xFNK exhibited high resistance to treatment with TN-16 and staurosporine, and the ~~dehydrogense~~ dehydrogenase activity was maintained at high level.

(b) Resistance to apoptosis induced by depletion of the cytokine IL-3

The cells were washed 3 times with PBS and suspended in culture medium containing no IL-3 (but containing serum) at about 5×10^4 cells/ml, and the surviving cells were counted by the trypan blue exclusion everyday. The result are shown in Fig. 16, in which the number of the surviving cells immediately after depletion of IL-3 was taken as 100%. In this experiment, the cells other than

Incubation of the CHO transfectants in serum-free medium

Three transfectants, CHO*bcl-x*, CHO*bcl-x*FNK, and CHO*vec*, prepared in Example 6 were incubated in culture medium DMEM/F-12 containing 10% fetal bovine serum. The cells (1×10^3 cells) were plated in a 100-mm dish containing the medium DMEM/F-12 containing 3% fetal bovine serum. For 5 consecutive days, two thirds of the culture medium was replaced with the DMEM/F-12 containing no fetal bovine serum. The cells were incubated on the medium lacking serum from day 6. The incubation was further continued for another 6 days.

The results are shown in the photograph of Fig. 17. CHO*bcl-x*FNK expressing the improved Bcl-xFNK (Fig. 17C) grew far better than CHO*vec* (Fig. 17B) to which the empty vector was introduced. Additionally, fewer cells were dying or dead and the cells more firmly contacted each other without space in CHO*bcl-x*FNK colonies than those in the colonies of CHO*bcl-x* cells expressing Bcl-x_L (Fig. 17A).

From the above results, it was confirmed that the transfected cells of the invention could grow well in a normal state even in a serum-free medium.

Example 8

Construction of a recombinant vector expressing the TAT-Bcl-xFNK protein

The engineered cDNA coding for Bcl-xFNK prepared in Example 1 was fused to the cDNA coding for the protein transduction domain of TAT protein of HIV virus by 2-step PCR. PCR was carried out using Primer 9 (SEQ ID NO: 14) as the 5'-end primer, Primer 10 (SEQ ID NO: 15) as the 3'-end primer and the recombinant vector pEF1-BOS*bcl-x*Y22F/Q26N/R165K having Bcl-xFNK cDNA as a template. Primer 9 is the sense sequence consisting of the 3'-end of TAT-PTD cDNA and the 5'-end (containing the initiation codon) of Bcl-xFNK cDNA. Primer 10 is the antisense sequence ~~consiting~~ consisting of the 3'-end (containing a termination

codon) of Bcl-xFNK cDNA and the cleavage site for the restriction enzyme *Hind* III. Details of the PCR reaction are as follows

· Reaction solution (volume 100 ~~ml~~ μl ← effected in Preliminary Amendment dated 2/19/02): 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM each dATP, dCTP, dTTP, dGTP, , AmpliTaqGOLD: 2.5U

· Primers: a ~~comibination~~ combination of Primer 9 and Primer 10 (each primer: 1 μM)

· Template DNA: 50 ng

· Reaction condition 3: 94°C/10 min (94°C/30 sec; 49°C/30 sec; 72°C/1 min) × 15 cycles.

After the reaction, the amplified DNA fragment was purified by polyacrylamide gel electrophoresis. To the above-described PCR reaction solution (25 μl) was added the purified DNA fragment (25 ng) and Primer 11 (SEQ ID NO: 16) to synthesize the complementary strand using AmpliTaqGOLD. Primer 16 is the 5'-end sense sequence coding for the amino acid sequence of TAT-PTD flanked by Met (initiation codon)-Gly at the 5'-end and Gly- the initiation codon of Bcl-xFNK cDNA at the 3'-end, as shown in SEQ ID NO: 12. The condition for synthesis is as follows.

· Reaction condition 4: 94°C/10 min (94°C/30 sec; 53°C to 59°C/30 sec; 72°C/1 min) × 5 cycles

After the reaction, the PCR solution (75 ~~ml~~ μl ← effected in Preliminary Amendment dated 2/19/02) containing Primer 12 (SEQ ID NO: 17), Primer 10 (final concentration: 1 μM each), and AmpliTaqGOLD (2.5 U) was added, and the PCR was carried out according to the above reaction condition 3. Primer 12 is a sense sequence coding for Met-Gly and the subsequent N-terminal three amino acid residues of TAT-PTD with a cleavage site of the restriction enzyme *Nde* I at the 5'-end. The amplified DNA fragment was purified by polyacrylamide gel electrophoresis. After cleavage with *Nde* I, the cleaved end was made blunt with T4DNA polymerase and further subjected to be digested with *Hind* III. The *Escherichia coli* expression vector pROEX1 (Life Technologies) was cleaved with *Nco* I, then made blunt with nuclease S1, followed by digestion with Hind III. Two DNAs were ligated to each other to yield the recombinant vector

pROEX1- -*bcl-x*Y22F/Q26N/R165K coding for TAT-Bcl-xFNK in which a protein-transduction-domain peptide of TAT protein is fused at the N-terminal.

Example 9

Preparation of TAT-Bcl-xFNK protein

TAT-Bcl-xFNK protein was expressed in *Escherichia coli* and partially purified as described below. *Escherichia coli* DH5αMCR carrying pROEX1-*bcl-x*Y22F/Q26N/R165K was incubated on 1000 ml of LB liquid medium (5 g yeast extract, 10 g Bactotrypton, and 5 g NaCl) containing ampicillin (50 μg/mg) with shaking at 37°C. When the cells reached at the logarithmic growth phase (O.D.600= 0.5), IPTG (isopropyl-1-thio-β-galactoside; final concentration 1 mM) was added, and the incubation was continued for 2 hours. TAT-Bcl-xFNK protein was prepared from a soluble fraction and an insoluble fraction (inclusion body) after disrupting the cells. The protein was prepared from the soluble fraction as follows. The harvested cells were washed 3 times with PBS, then suspended in 40 ml of Buffer A (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF), and disrupted by ultrasonication. After centrifugation, TAT-Bcl-xFNK was purified from the supernatant by antibody-affinity chromatography using a column in which was packed a monoclonal antibody 35-32 bound to carriers, said monoclonal antibody being derived from a mouse and recognizing the N-terminal region of rat Bcl-x_L. TAT-Bcl-xFNK was bound to the antibody, washed, and then eluted with an eluate (50 mM Glycine-HCl pH 2.7, 50 mM NaCl). The eluate was neutralized with 2M Tris-HCl (pH 9.0) and concentrated by Centricon (Amicon). Dialysis against PBS gave TAT-Bcl-xFNK preparation for use in the following experiment. TAT-Bcl-xFNK was prepared from the insoluble fraction (inclusion body) as follows. The harvested cells were washed 3 times with PBS, then suspended in 36 ml of Buffer A (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT) containing DTT in place of PMSF,

cell death.

Example 12

Administration of TAT-Bcl-xFNK to mice and confirmation of the inhibition of the death of hepatocytes caused by steroid hormone

Three 8-week old mice (C56BL, 20 g body weight, female) were divided into 3 groups (A, B and C). To the mouse of Group A was intraperitonealy administered PBS solution (0.8 ml) containing 100 μg of TAT-Bcl-xFNK protein (prepared from the soluble fraction). To the mice of Group B and Group C (control) was intraperitonealy administered PBS (0.8 ml) in the same manner. The mice were put back into cages, and after a lapse of 3 hours 0.5 ml of 25% ethanol/PBS solution containing 0.5 mg of a steroid hormone (dexamethasone) was intraperitonealy administered to the mice of Group A and Group B. To the mouse of Group C was intraperitonealy administered 0.5 ml of 25% ethanol/PBS solution. They were put back into cages, and after a lapse of 3 hours killed. Their livers were taken out, and frozen to prepare frozen sections with a cryostat. The sections were stained with hematoxylin-eosin to evaluate the death of hepatocyte. Degeneration of the hepatic tissue and cell death caused by dexamethasone in Group B as shown in Fig. 26 were markedly inhibited by pre-administration of TAT-Bcl-xFNK (Group A; Fig. 25). The degree was shown to be better than that of the control (Group C; Fig. 27).

The above results indicate that the protein TAT-Bcl-xFNK intraperitoneally administered is delivered into the hepatic cells to strongly inhibit cell death caused by dexamethasone.

~~**Industrial Applicability**~~

CLAIMS

What is claimed is:

1. A genetically engineered cDNA of the rat *bcl-x* gene, which has at least one substitution selected from the substitutions that change residues 22 Tyr to Phe, residues 26 Gln to Asn and residues 165 Arg to Lys, in the coding region of rat *bcl-x* cDNA of SEQ ID NO: 1.

2. The genetically engineered cDNA of claim 1, which is attached at its 5'-end with an oligonucleotide encoding a protein-transduction-domain peptide.

3. The genetically engineered cDNA of claim 2, wherein the oligonucleotide encodes the amino acid sequence of SEQ ID NO: 12 or 13.

4. A recombinant vector carrying the genetically engineered cDNA of any one of claims 1 to 3.

5. A cell into which the recombinant vector of claim 4 was introduced.

6. An improved protein produced by the genetically engineered cDNA of claim 1, which has at least one amino acid substitution in SEQ ID NO: 2, which amino acid substitution is selected from the substitutions of residues 22 Tyr to Phe, residues 26 Gln to Asn and residues 165 Arg to Lys.

7. The improved protein of claim 6, which is attached at the N-terminal with a protein-transduction-domain peptide.

8. The improved protein of claim 7, wherein the protein-transduction-domain peptide is an oligopeptide having the amino acid sequence of SEQ ID NO: 12 or 13.

ABSTRACT OF THE DISCLOSURE

The present invention provides an genetically engineered cDNA of the rat *bcl-x* gene, which has at least one substitution selected from the substitutions that change residues 22 Tyr to Phe, residues 26 Gln to Asn and residues 165 Arg to Lys, in the coding region of rat *bcl-x* cDNA of SEQ ID NO: 1, a recombinant vector containing the engineered cDNA, a cell into which the recombinant vector was introduced, and an improved protein of Bcl-x_L. The improved protein of Bcl-x_L is useful as an ingredient for remedies for various diseases accompanied with cell death since it effectively inhibit cell death such as apoptosis.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	:	**Confirmation No. 6325**
Shigeo OHTA et al.	:	Docket No. 2002-0256A
Serial No. 10/049,822	:	Group Art Unit Not Yet Assigned
Filed April 1, 2002	:	Examiner Not Yet Assigned
A GENETICALLY ENGINEERED cDNA OF RAT bcl-x GENE AND AN IMPROVED PROTEIN	:	

THE COMMISSIONER IS AUTHORIZED TO CHARGE ANY DEFICIENCY IN THE FEES FOR THIS PAPER TO DEPOSIT ACCOUNT NO. 23-0975

RESPONSE

Assistant Commissioner for Patents,
Washington, D.C. 20231

Sir:

Responsive to the Notice dated May 30, 2002, there is submitted herewith, in a separate Preliminary Amendment, a paper copy of a Sequence Listing for the above-identified application which has been prepared in accordance with the sequence rules under 37 CFR 1.821-1.825. The Sequence Listing contains the identical sequences appearing in the original application papers. Thus, no new matter has been added.

There is also submitted herewith a copy of the Sequence Listing in computer readable form as required by 37 CFR 1.821(e). The content of the paper and computer readable copies are the same.

A copy of the Notice is also attached as required.

In view of the foregoing, it is believed that each requirement set forth in the Notice has been satisfied, and that the application is now in compliance with the sequence rules under 37 CFR 1.821-1.825. Accordingly, favorable examination on the merits is respectfully requested.

Respectfully submitted,

Shigeo OHTA et al.

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June 25, 2002

SEQUENCE LISTING

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DT05 Rec'd PCT/PTO 06 SEP 2002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	:	**Confirmation No. 6325**
Shigeo OHTA et al.	:	Docket No. 2002-0256A
Serial No. 10/049,822	:	Group Art Unit Not Yet Assigned
Filed April 1, 2002	:	Examiner Not Yet Assigned
A GENETICALLY ENGINEERED cDNA OF RAT bc1-x GENE AND AN IMPROVED PROTEIN	:	

THE COMM[illegible] AUTHORIZED TO CHARG[illegible] [illegible]FICIENCY IN THE FEES FOR THI[illegible]ER TO DEPOSIT ACCOUNT NO 2[illegible]0975

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents,
Washington, D.C. 20231

Sir:

Responsive to the Notice dated August 29, 2002, please amend the above-identified application as follows:

In the Sequence Listing:

Please replace the Sequence Listing of record with the attached substitute Sequence Listing.

REMARKS

The foregoing amendments are presented to place the application in compliance with the sequence rules under 37 CFR 1.821-1.825.

Applicants have submitted a revised Sequence Listing in both paper and computer readable form as required by 37 C.F.R. 1.821(c) and (e). Amendments directing its entry into the specification have also been incorporated herein. The content of the paper and computer readable copies are the same and no new matter has been added.

In view of the foregoing, it is believed that each requirement set forth in the Notice has been satisfied, and that the application is now in compliance with the sequence rules under 37 CFR 1.821-1.825. Accordingly, favorable examination on the merits is respectfully requested.

Respectfully submitted,

Shigeo OHTA et al.

By:_____

Lee Cheng
Registration No. 40,949
Attorney for Applicants

LC/gtg
Washington, D.C. 20006-1021
Telephone (202) 721-8200
Facsimile (202) 721-8250
September 6, 2002

OIPE JC64
SEP 06 2002
PATENT & TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	:	**Confirmation No. 6325**
Shigeo OHTA et al.	:	Docket No. 2002-0256A
Serial No. 10/049,822	:	Group Art Unit Not Yet Assigned
Filed April 1, 2002	:	Examiner Not Yet Assigned
A GENETICALLY ENGINEERED cDNA OF RAT bcl-x GENE AND AN IMPROVED PROTEIN	:	

THE COMMISSIONER IS AUTHORIZED TO CHARGE ANY DEFICIENCY IN THE FEES FOR THIS PAPER TO DEPOSIT ACCOUNT NO. 23-0975

RESPONSE

Assistant Commissioner for Patents,
Washington, D.C. 20231

Sir:

Responsive to the Notice dated August 29, 2002, there is submitted herewith, in a separate Preliminary Amendment, a paper copy of a revised Sequence Listing for the above-identified application which has been prepared in accordance with the sequence rules under 37 CFR 1.821-1.825. The revised Sequence Listing contains the identical sequences appearing in the original application papers. Thus, no new matter has been added.

There is also submitted herewith a copy of the revised Sequence Listing in computer readable form as required by 37 CFR 1.821(e). The content of the paper and computer readable copies are the same.

A copy of the Notice is also attached as required.

In view of the foregoing, it is believed that each requirement set forth in the Notice has been satisfied, and that the application is now in compliance with the sequence rules under 37 CFR 1.821-1.825. Accordingly, favorable examination on the merits is respectfully requested.

Respectfully submitted,

Shigeo OHTA et al.

By: _____
Lee Cheng
Registration No. 40,949
Attorney for Applicants

LC/gtg
Washington, D.C. 20006-1021
Telephone (202) 721-8200
Facsimile (202) 721-8250
September 6, 2002

OIPE JC64
SEP 0 6 2002
PATENT & TRADEMARK OFFICE

SEQUENCE LISTING

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ASOH, Sadamitsu

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<303> J. Biol. Chem.
<304> 271
<305> 22
<306> 13258-13265
<307> 1996-05-31

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JC13 Rec'd PCT/PTO 19 FEB 2002

DESCRIPTION

A Genetically Engineered cDNA of Rat *bcl-x* Gene and An Improved Protein

this application is a 371 of PCT/JP00/05502 filed August 17, 2000.

Technical Field

The present invention relates to a genetically engineered cDNA of rat *bcl-x* gene and an improved protein. More particularly, it relates to a novel cDNA expressing an improved protein of Bcl-x_L having higher apoptosis-inhibiting activity and cell death-inhibiting activity than the protein Bcl-x_L expressed by the rat's apoptosis-inhibiting gene *bcl-x*. The invention also relates to materials for utilizing such cDNA in gene engineering, as well as to an improved protein of Bcl-x_L expressed by the cDNA.

Background Art

Apoptosis is one of programmed cell death. Apoptosis is accompanied by poor contact with the surrounding cells, concentration of cytoplasm, condensation of the chromatin and nuclei associated with the endonuclease activity, fragmentation of the nuclei, formation of membrane-bounded apoptotic bodies, and phagocytosis of the apoptotic bodies by the adjacent macrophage or epithelial cells. A phenomenon that the chromosomal DNA is cleaved into DNA fragments of 180 to 200 base length by the endonuclease activity is also observed. Such phenomena have been discussed as the mechanism indicating that the apoptotic bodies are finally phagocytosed by the adjacent cells (for example, Immunology Today 7:115-119, 1986; Science 245:301-305, 1989).

As a gene controlling the apoptosis, for example, the gene *bcl-2* which is

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Fig. 16 shows the results of a test for resistance of the transfected cells to apoptosis induced by depletion of the cytokine IL-3.

Fig. 17 is a microscopic photograph showing the transfected CHO cells growing in a serum-free medium.

Fig. 18 is a microscopic photograph showing a state of the protein TAT-Bcl-xFNK incorporated in the HeLa cells.

Fig. 19 is a microscopic photograph of the chondrocytes in cartilage slice incubated in a culture medium containing the protein TAT-Bcl-xFNK for 5 days.

Fig. 20 is a microscopic photograph of the chondrocytes in cartilage slice incubated in a culture medium containing the protein TAT-Bcl-xFNK for 9 days.

Fig. 21 is a microscopic photograph of the chondrocytes in cartilage slice incubated in a culture medium containing the protein ~~Bcl-xFNK~~ TAT-Bcl-xL for 5 days.

Fig. 22 is a microscopic photograph of the chondrocytes in cartilage slice incubated in a culture medium containing the protein ~~Bcl-xFNK~~ TAT-Bcl-xL for 9 days.

Fig. 23 is a microscopic photograph of the chondrocytes in cartilage slice incubated in a culture medium containing a solvent (PBS) for 5 days.

Fig. 24 is a microscopic photograph of the chondrocytes in cartilage slice incubated in a culture medium containing a solvent (PBS) for 9 days.

Fig. 25 is a microscopic photograph showing the liver slice of a mouse to which the protein TAT-Bcl-xFNK has been administered systemically, followed by

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codon) of Bcl-xFNK cDNA and the cleavage site for the restriction enzyme *Hind* III. Details of the PCR reaction are as follows

· Reaction solution (volume ~~100 ml~~ 100 µl): 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM each dATP, dCTP, dTTP, dGTP, , AmpliTaqGOLD: 2.5U

· Primers: a comibination of Primer 9 and Primer 10 (each primer: 1 µM)

· Template DNA: 50 ng

· Reaction condition 3: 94°C/10 min (94°C/30 sec; 49°C/30 sec; 72°C/1 min) × 15 cycles

After the reaction, the amplified DNA fragment was purified by polyacrylamide gel electrophoresis. To the above-described PCR reaction solution (25 µl) was added the purified DNA fragment (25 ng) and Primer 11 (SEQ ID NO: 16) to synthesize the complementary strand using AmpliTaqGOLD. Primer 16 is the 5'-end sense sequence coding for the amino acid sequence of TAT-PTD flanked by Met (initiation codon)-Gly at the 5'-end and Gly- the initiation codon of Bcl-xFNK cDNA at the 3'-end, as shown in SEQ ID NO: 12. The condition for synthesis is as follows.

· Reaction condition 4: 94°C/10 min (94°C/30 sec; 53°C to 59°C/30 sec; 72°C/1 min) × 5 cycles

After the reaction, the PCR solution (~~75 ml~~ 75 µl) containing Primer 12 (SEQ ID NO: 17), Primer 10 (final concentration: 1 µM each), and AmpliTaqGOLD (2.5 U) was added, and the PCR was carried out according to the above reaction condition 3. Primer 12 is a sense sequence coding for Met-Gly and the subsequent N-terminal three amino acid residues of TAT-PTD with a cleavage site of the restriction enzyme *Nde* I at the 5'-end. The amplified DNA fragment was purified by polyacrylamide gel electrophoresis. After cleavage with *Nde* I, the cleaved end was made blunt with T4DNA polymerase and further subjected to be digested with *Hind* III. The *Escherichia coli* expression vector pROEX1 (Life Technologies) was cleaved with *Nco* I, then made blunt with nuclease S1, followed by digestion with Hind III. Two DNAs were ligated each other to yield the recombinant vector

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pROEX1- -*bcl-x*Y22F/Q26N/R165K coding for TAT-Bcl-xFNK in which a protein-transduction-domain peptide of TAT protein is fused at the N-terminal.

Example 9

Preparation of TAT-Bcl-xFNK protein

TAT-Bcl-xFNK protein was expressed in *Escherichia coli* and partially purified as described below. *Escherichia coli* DH5αMCR carrying pROEX1-*bcl-x*Y22F/Q26N/R165K was incubated on 1000 ml of LB liquid medium (5 g yeast extract, 10 g Bactotrypton, and 5 g NaCl) containing ampicillin (50 μg/mg) with shaking at 37°C. When the cells reached at the logarithmic growth phase (O.D.600≈ 0.5), IPTG (isopropyl-1-thio-β-galactoside; final concentration 1 mM) was added, and the incubation was continued for 2 hours. TAT-Bcl-xFNK protein was prepared from a soluble fraction and an insoluble fraction (inclusion body) after disrupting the cells. The protein was prepared from the soluble fraction as follows. The harvested cells were washed 3 times with PBS, then suspended in 40 ml of Buffer A (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF), and disrupted by ultrasonication. After centrifugation, TAT-Bcl-xFNK was purified from the supernatant by antibody-affinity chromatography using a column in which was packed a monoclonal antibody 35-32 bound to carriers, said monoclonal antibody being derived from a mouse and recognizing the N-terminal region of rat Bcl-x_L. TAT-Bcl-xFNK was bound to the antibody, washed, and then eluted with an eluate (50 mM Glycine-HCl pH 2.7, 50 mM NaCl). The eluate was neutralized with 2M Tris-HCl (pH 9.0) and concentrated by Centricon (Amicon). Dialysis against PBS gave TAT-Bcl-xFNK preparation for use in the following experiment. TAT-Bcl-xFNK was prepared from the insoluble fraction (inclusion body) as follows. The harvested cells were washed 3 times with PBS, then suspended in 36 ml of Buffer A (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT) containing DTT in place of PMSF,

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CLAIMS

1. A genetically engineered cDNA of the rat *bcl-x* gene, which has at least one substitution selected from the substitutions that change residues 22 Tyr to Phe, residues 26 Gln to Asn and residues 165 Arg to Lys, in the coding region of rat *bcl-x* cDNA of SEQ ID NO: 1.

2. The genetically engineered cDNA of claim 1, which is attached at its 5'-end with an oligonucleotide encoding a protein-transduction-domain peptide.

3. The genetically engineered cDNA of claim 2, wherein the oligonucleotide encodes the amino acid sequence of SEQ ID NO: 12 or 13.

4. (Amended) A recombinant vector carrying the genetically engineered cDNA of ~~any one of claims~~ 1 ~~to 3~~.

5. A cell into which the recombinant vector of claim 4 was introduced.

6. An improved protein produced by the genetically engineered cDNA of claim 1, which has at least one amino acid substitution in SEQ ID NO: 2, which amino acid substitution is selected from the substitutions of residues 22 Tyr to Phe, residues 26 Gln to Asn and residues 165 Arg to Lys.

7. The improved protein of claim 6, which is attached at the N-terminal with a protein-transduction-domain peptide.

8. The improved protein of claim 7, wherein the protein-transduction-domain peptide is an oligopeptide having the amino acid sequence of SEQ ID NO: 12 or 13.

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17/prts

DESCRIPTION

A Genetically Engineered cDNA of Rat *bcl-x* Gene and An Improved Protein

Technical Field

The present invention relates to a genetically engineered cDNA of rat *bcl-x* gene and an improved protein. More particularly, it relates to a novel cDNA expressing an improved protein of Bcl-x_L having higher apoptosis-inhibiting activity and cell death-inhibiting activity than the protein Bcl-x_L expressed by the rat's apoptosis-inhibiting gene *bcl-x*. The invention also relates to materials for utilizing such cDNA in gene engineering, as well as to an improved protein of Bcl-x_L expressed by the cDNA.

Background Art

Apoptosis is one of programmed cell death. Apoptosis is accompanied by poor contact with the surrounding cells, concentration of cytoplasm, condensation of the chromatin and nuclei associated with the endonuclease activity, fragmentation of the nuclei, formation of membrane-bounded apoptotic bodies, and phagocytosis of the apoptotic bodies by the adjacent macrophage or epithelial cells. A phenomenon that the chromosomal DNA is cleaved into DNA fragments of 180 to 200 base length by the endonuclease activity is also observed. Such phenomena have been discussed as the mechanism indicating that the apoptotic bodies are finally phagocytosed by the adjacent cells (for example, Immunology Today 7:115-119, 1986; Science 245:301-305, 1989).

As a gene controlling the apoptosis, for example, the gene *bcl-2* which is

ATTACHMENT A

an proto-oncogene found in the human follicular B cell lymphoma is known (Science 226 (4678): 1097-1099, 1984; Pro. Natl. Acad. Sci. USA 81(22): 7166-7170, 1984). Analysis of the gene structure and the transcripts or the cDNA clones have been also reported (Pro. Natl. Acad. Sci. USA 83(14): 5214-5218, 1986; Cell 47(1): 19-28, 1886). This *bcl-2* gene is expressed in immune and nervous systems with high frequency. The gene product is considered to maintain homeostasis of the human immune and nervous systems by inhibiting apoptosis of the cells. Moreover, the *bcl-2* gene is also considered to play an important role in morphogenesis during development since it is widely expressed particularly in the fetus

Thereafter, homologues of the gene *bcl-2* were found in bovine, rat, chicken, etc., and they are collectively called the *bcl-2* family.

The inventors of the present application have cloned rat *bcl-x* gene as a homologue of the human *bcl-x* gene (Cell 74(4): 597-608, 1993) belonging to the *bcl-2* family (J. Biol. Chem. 271(22): 13258-13265, 1996). They have also determined the three-dimensional structure of the Bcl-x_L protein expressed from the rat *bcl-x* gene by X-ray analysis (J. Biol. Chem. 272(44): 27886-27892, 1997).

The inventors of the present application investigated the substitutions of amino acid residue causing conformational change to enhance the anti-apoptotic activity of rat Bcl-x_L. They genetically engineered cDNA of the *bcl-x* gene to replace a specific amino acid residue with other amino acid residue, and finally obtained the engineered cDNA whose product markedly inhibited cell death involving apoptosis.

The invention of the present application was completed based on these new findings by the inventors. The purpose of the invention is to provide the engineered cDNA that allows expression of this novel improved protein of rat

Bcl-x_L in cells.

Another purpose of the invention is to provide a recombinant vector containing this engineered cDNA and a cell having the recombinant vector.

Still another purpose of the invention is to provide the improved protein expressed from the above-described engineered cDNA.

Disclosure of Invention

In order to solve the above-described problems, the present application provides the following inventions (1) to (8).

(1) A genetically engineered cDNA of the rat *bcl-x* gene, which has at least one substitution selected from the substitutions that change residues 22 Tyr to Phe, residues 26 Gln to Asn and residues 165 Arg to Lys, in the coding region of rat *bcl-x* cDNA of SEQ ID NO: 1.

(2) The genetically engineered cDNA of said invention (1), which is attached at its 5'-end with an oligonucleotide encoding a protein-transduction-domain peptide.

(3) The genetically engineered cDNA of said invention (2), wherein the oligonucleotide encodes the amino acid sequence of SEQ ID NO: 12 or 13.

(4) A recombinant vector carrying the genetically engineered cDNA of any one of said inventions (1) to (3).

(5) A cell into which the recombinant vector of said invention (4) is

introduced.

(6) An improved protein produced from the genetically engineered cDNA of said invention (1), which has at least one amino acid substitution in SEQ ID NO: 2, which the amino acid substitution is selected from the substitutions of residues 22 Tyr with Phe, reisdues 26 Gln with Asn and residues 165 Arg with Lys.

(7). The improved protein of said invention (6), which is attached at the N-terminal with a protein-transduction-domain peptide.

(8). The improved protein of said invention (7), wherein the protein-transduction-domain peptide is an oligopeptide having the amino acid sequence of SEQ ID NO: 12 or 13.

Brief Description of Drawings

Fig. 1 shows the three-dimensional structure of the wild-type rat Bcl-x_L.

Fig. 2 shows the results of Western blotting analysis of the expression level of the improved protein Bcl-xLFNK in the transfected cell.

Fig. 3 shows the results of a test for resistance of the transfected cells to apoptosis induced by serum depletion.

Fig. 4 shows the results of a test for resistance of the transfected cells to anti-Fas antibody.

Fig. 5 shows the results of a test for resistance of the transfected cells to staurosporine.

Fig. 6 shows the results of a test for resistance of the transfected cells to TN-16.

Fig. 7 shows the results of a test for resistance of the transfected cells to camptothecin.

Fig. 8 shows the results of a test for resistance of the transfected cells to hydroxyurea.

Fig. 9 shows the results of a test for resistance of the transfected cells to trichostatin A.

Fig. 10 shows the results of a test for resistance of the transfected cells to hydrogen peroxide.

Fig. 11 shows the results of a test for resistance of the transfected cells to paraquat.

Fig. 12 shows the results of a test for resistance of the transfected cells to heat. treatment

Fig. 13 shows the results of a test for dehydrogenase activity in the transfected cells after heat treatment by the WST-1 assay.

Fig. 14 shows the results of a test for dehydrogenase activity in the transfected cells treatmented with TN-16 by the WST-1 assay.

Fig. 15 shows the results of a test for dehydrogenase activity in the transfected cells treatmented with staurosporine by the WST-1 assay.

Fig. 16 shows the results of a test for resistance of the transfected cells to apoptosis induced by depletion of the cytokine IL-3.

Fig. 17 is a microscopic photograph showing the transfected CHO cells growing in a serum-free medium.

Fig. 18 is a microscopic photograph showing a state of the protein TAT-Bcl-xFNK incorporated in the HeLa cells.

Fig. 19 is a microscopic photograph of the chondrocytes in cartilage slice incubated in a culture medium containing the protein TAT-Bcl-xFNK for 5 days.

Fig. 20 is a microscopic photograph of the chondrocytes in cartilage slice incubated in a culture medium containing the protein TAT-Bcl-xFNK for 9 days.

Fig. 21 is a microscopic photograph of the chondrocytes in cartilage slice incubated in a culture medium containing the protein Bcl-xFNK for 5 days.

Fig. 22 is a microscopic photograph of the chondrocytes in cartilage slice incubated in a culture medium containing the protein Bcl-xFNK for 9 days.

Fig. 23 is a microscopic photograph of the chondrocytes in cartilage slice incubated in a culture medium containing a solvent (PBS) for 5 days.

Fig. 24 is a microscopic photograph of the chondrocytes in cartilage slice incubated in a culture medium containing a solvent (PBS) for 9 days.

Fig. 25 is a microscopic photograph showing the liver slice of a mouse to which the protein TAT-Bcl-xFNK has been administered systemically, followed by

administration of dexamethasone.

Fig. 26 is a microscopic photograph showing the liver slice of a mouse to which a solvent (PBS) has been administered systemically, followed by administration of dexamethasone.

Fig. 27 is a microscopic photograph showing the liver slice of a mouse to which a solvent (PBS) only has been administered systemically.

The Best Mode for Carrying Out the Invention

The engineered cDNA of the invention as indicated in the above invention (1), is characterized by having at least one substitution selected from the substitutions that change the codon of residues 22 Tyr (tac) to the codons of Phe (ttt/ttc), the codon of residues 26 Gln (cag) to the codons of Asn (aat/aac) and the codon of residues 165 Arg (cgg) to the codons of Lys (aaa/aag), in the coding region of rat *bcl-x* cDNA of SEQ ID NO: 1. In a preferred embodiment, the nucleotide substitutions have occurred in all the above 3 sites in the engineered cDNA of the invention (1). The engineered cDNA which has the nucleotide substitutions of at the 3 sites produces the improved protein Bcl-xFNK as shown in the amino acid sequence of SEQ ID NO: 3. In this improved protein Bcl-xFNK, three hydrogen bonds between Tyr^{22} and Asp^{156}, between Gln^{26} and Ser^{164} and between Arg^{165} and Pro^{116}, which are formed in the wild-type rat Bcl-x_L as shown in the three-dimensional structure of Fig. 1, are disturbed as a result of the amino acid substitutions (Tyr22Phe; Gln26Asn; Arg165Lys) caused by the above-described nucleotide substitutions.

The engineered cDNA may be prepared by a known method with a mutation kit or a PCR method as mentioned in Examples using the rat *bcl-x* cDNA

as a template. For the cDNA of rat *bcl-x*, the plasmid pEF1-BOS*bcl-x* (J. Biol. Chem. 271(22): 13258-13265, 1996) can be used. An alternative method may be used, in which an oligonucleotide of any parts of the nucleotide sequence of SEQ ID NO: 1 is synthesized to use as a probe for screening of a rat cDNA library. Or an oligonucleotide that hybridizes with both ends of the cDNA fragment to be a subject may be synthesized and used as a primer in an RT-PCR method of preparing the cDNA from the mRNA isolated from rat cells.

The inventions (2) and (3) relates to a DNA fragment (polynucleotide) in which an oligonucleotide encoding a protein-transduction-domain peptide is ligated to the 5'-end of the engineered cDNA of the above invention (1). This DNA fragment can be used in preparation of the improved protein of Bcl-x_L as mentioned below.

The recombinant vector in the invention (4) of the present application may be prepared by choosing an appropriate expression vector depending on a type of cell to be introduced (for example, prokaryotic cells such as *Escherichia coli* or *Bacillus subtilis*; eukaryotic cells such as yeast, insect cells, mammalian cells, or plant cells) and integrating therein the engineered cDNA of any one of the inventions (1) to (3). For example, when a microorganism such as *Escherichia coli* is employed, any of the engineered cDNAs of the above inventions (1) to (3) is integrated into the DNA cloning site of an expression vector having an replication origin functioning in a microorganism, promoter, ribosome-binding site, terminator, etc. When a eukaryotic cell such as a mammalian cell is employed, a recombinant vector of the invention (4) may be prepared using an expression vector for eukaryotic cells having a promoter, splice sites, a poly(A) site, etc.

The cell of the invention (5) is a cell into which the recombinant vector of the invention (4) is introduced and which produces the improved protein of Bcl-x_L. There is no limitation in the type of cell to be used. The recombinant vector of the

invention (4) can be introduced into all of the cells, for example, prokaryotic cells such as *Escherichia coli* or *Bacillus subtilis*; eukaryotic cells such as yeast, insect cells, mammalian cells, or plant cells are included. Introduction of the recombinant vector into cells may be achieved by a known method. For example, when the recombinant vector is introduced into a mammalian cell, electroporation, a calcium phosphate method, a liposome method, a DEAE dextran method, and the like may be employed.

Among the cells of the invention (5), particularly, the mammalian cells can be also proliferated in a serum-free medium as shown in the data of Examples below. In general, in order to keep a cultured cell alive for a certain period of time, it is necessary to add a serum (e.g., fetal bovine serum) containing growth factors to the culture medium. Addition of the growth factors can inhibit apoptosis of the cells to prolong the cellular life span. When cellular products such as physiologically active substances or monoclonal antibodies are recovered and purified from the culture medium in which mammalian cells are grown, however, it is desirous that the culture medium contains no impurities such as serum. The reason is that the cost increases and extra steps are required for purifying the objective substance, and that there is a possibility of the serum containing a risk factor such as virus. The use of the serum-free and protein-free medium containing no serum, however, practically reduces the degree of cell growth and results in increasing dead cells, when the serum-free and protein-free media have been used. In addition, there is a problem that such increased dead cells might cause contamination of the celluar contents into the culture medium because of an outflow of the cellular contents from the dead cells.

On the other hand, there is an alternative method for proliferating cells without using any growth factor, wherein the cells are transfected with an proto-oncogene. In this method, however, it has been elucidated that apoptosis is rather promoted by expression of multiple proto-oncogene products.

The transfected mammalian cells of the invention (5) can be cultivated over a long period of time without accompanying apoptosis even in the absence of any growth factor such as serum since they express the improved protein of Bcl-x_L. Based on such excellent growth ability, it is possible to establish a cell line.

The improved protein of Bcl-x_L of the invention (6) is expressed from the engineered cDNA of the invention (1). The protein is characterized by having at least one amino acid substitution in SEQ ID NO: 2, which substitution is selected from the substitutions of Tyr^{22} with Phe, Gln^{26} with Asn and Arg^{165} with Lys. Bcl-xFNK that has the amino acid sequence of SEQ ID NO: 3 containing all of the amino acid substitutions as described above, is the most preferred embodiment..

In producing the improved proteins, the cells of the invention (5) are cultured to yield materials of culture, from which the improved proteins can be isolated and purified by combined known methods for isolation. The known methods for isolation include, for example, treatment with a denaturant or surface activator such as urea, ultra-sonication, digestion with an enzyme, salting-out or precipitation with a solvent, dialysis, centrifugation, ultra-filtration, gel filtration, SDS-PAGE, isoelectric focusing, ion exchange chromatography, hydrophobic chromatography, affinity chromatography, reverse phase chromatography, and the like.

The improved proteins can be used, for example, as active ingredients in apoptosis inhibitors or their leading compounds. Moreover, it is preferable to bind a protein-transduction-domain peptide to the N-terminal of the improved proteins of Bcl-x_L. The improved protein of Bcl-x_L having the protein-transduction-domain peptide traverse the cell membrane and enter the cell to exhibit transiently a function of inhibiting apoptosis and cell death. Thus, the improved proteins acquiring such ability to traverse cell membranes can be

utilized, for example in the following uses.

(a) To maintain cells to be used for implantation in a normal state over a long period of time.

(b) To maintain organs to be used for transplantation of organs in a normal state over a long period of time.

(c) To maintain organs subjected to hemostasis in a stable state during a surgical operation

(d) To use as a therapeutic for cell death caused by cerebral ischemia accompanying cerebral thrombosis etc.

(e) To use as a therapeutic for fulminant hepatitis.

(f) To use as a preventive for cell death caused by excess administration of steroid hormones.

(g) To use as a therapeutic for diseases accompanied by muscular atrophy (e.g., muscular dystrophy, myasthenia, myopathy, etc.) caused by death of myocytes.

(h) To use as a preventive for the death of skin epithelial cells caused by injury or burn.

For a protein-transduction-domain peptides, an oligopeptide having the amino acid sequence as shown in SEQ ID NO: 12 or SEQ ID NO: 13 can be used. The oligopeptide of SEQ ID NO: 12 is PTD (protein transduction domain) of HIV-1 TAT. The oligopeptide of SEQ ID NO: 13 is PTD of a homeobox protein, Antennapedia, of *drosophila*.

With respect to these protein-transduction-domain peptides, for example, the amino acid sequence and cDNA sequence of HIV-1 TAT are known (Science, 285:1569-1572, 1999; GenBank Accession NO. U39362 M96155). The DNA fragment encoding the region (47th to 57th amino acid sequence of HIV TAT) corresponding to the PTD is ligated to the engineered cDNA of the invention (1) to

give a fused DNA fragment (the invention (3)), which can be then expressed in a host cell such as *Escherichia coli* to produce the improved protein of Bcl-x_L having the PTD peptide at the N-terminal. Antennapedia PTD is also known (e.g., GenBank Accession No. AE001573) and can be used to construct the PTD-fused improved protein in a similar manner. Alternatively, the improved protein of Bcl-x_L is bound to a PTD peptide using a bivalent crosslinking agent (e.g., EDC or β-alanine) to construct the improved protein of Bcl-x_L bound to a protein-transduction-domain peptide.

Examples

The following examples serve to illustrate the invention specifically in more detail, but they are not intended to limit the scope of the invention.

Example 1

Preparation of genetically engineered cDNA

Two DNA fragments (*bcl-x*R165K, *bcl-x*Y22F/Q26N) were generated by two-step PCR using as a template the cDNA clone of rat Bcl-x_L, pEF1-BOS*bcl-x* (J. Biol. Chem. 271:13258-13265, 1996). Finally, these DNA fragments were linked at the given regions to yield an engineered cDNA *bcl-x*FNK containing 3 amino acid substitutions (Tyr22Phe; Gln26Asn; Arg165Lys).

First, in order to construct *bcl-x*R165K containing the substitution of Arg165Lys, two DNA fragments (A and B) were synthesized by PCR. For the DNA fragment (A), the primer 1 shown in SEQ ID NO: 4 was used as the 5'-end primer, and the primer 2 shown in SEQ ID NO: 5 as the 3'-end primer. The primer 1 consists of the nucleotide sequence of the vector and the nucleotide sequence of the upstream of the coding region of *bcl-x* cDNA. It also contains the cleavage

site of the restriction enzyme *Bam*H I. The primer 2 is an antisense sequence of *bcl-x* cDNA, in which the codon of Arg^{165} is substituted so as to code for Lys.

For the DNA fragment (B), the primer 3 shown in SEQ ID NO: 6 was used as the 5'-end primer, and the primer 4 shown in SEQ ID NO: 7 as the 3'-end primer. The primer 3 is a sense sequence of *bcl-x* cDNA, in which the codon of Arg^{165} is substituted so as to code for Lys, and the nucleotide sequence of 5'-end half is complementary to that of 5'-end half of the primer 2. The primer 4 is an antisense sequence of *bcl-x* cDNA, which corresponds to the amino acid residues 178 to 184 of the coding region. It also contains the cleavage site of the restriction enzyme *Bam*H I. PCR was carried out in the following conditions.

· Reaction solution (volume 100 μl): 10mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM each dATP, dCTP, dTTP, dGTP,

· AmpliTaqGOLD: 2.5U

· A pair of primers: a combination of Primer 1 and Primer 2, and a combination of Primer 3 and Primer 4 (each primer: 1μM)

· Template DNA: 50 ng

· Reaction condition 1: 94°C/10 min; (94°C/30 sec; 53°C/30 sec; 72°C/1 min)x15 cycles

After the reaction, the two amplified DNA fragments (A and B) were purified by polyacrylamide gel electrophoresis. Then, the DNA fragments A and B (6 ng each) were added to the above-described PCR reaction solution (25μl) to synthesize the respective complementary strands using AmpliTaqGOLD. The following reaction condition 2 was employed for the synthesis.

· Reaction condition 2: 94°C/10 min; (94°C/30 sec; 41°C to 47°C/30 sec; 72°C/1 min) x 4 cycles

After the reaction, a PCR reaction solution (75 μl) containing Primer 1, Primer 4 (final concentration: 1 μM each) and AmpliTaqGOLD (2.5U) were added, and the PCR was carried out according to the above reaction condition 1. The 650-bp PCR product was purified by polyacrylamide gel electrophoresis and then treated with the restriction enzyme *Bam*H I. On the other hand, pEF1-BOS*bcl-x*

(having two *Bam*H I sites) was treated with *Bam*H I to yield two DNA fragments (5650 bp and 650 bp). The longer DNA fragment (5650 bp) was ligated to the above PCR product in a right orientation to yield the clone pEF1-BOS*bcl-x*R165K having an amino acid substitution of Arg165Lys.

To construct *bcl-x*Y22F/Q26N, Gln^{26} was first substituted with Asn, followed by the amino acid substitution of Tyr^{22} with Phe. PCR was carried out using pEF1-BOS*bcl-x* (50 ng) as a template and a pair of the above Primer 1 and Primer 5 (SEQ ID NO:8). Another PCR was carried out using pEF1-BOS*bcl-x* (50 ng) as a template and a pair of the above Primer 4 and Primer 6 (SEQ ID NO:9). The components of the reaction solution (100μl) were the same as above, and the reactions were performed according to the above-described condition 1. Primer 5 is the antisense sequence of *bcl-x*cDNA and contains the nucleotide substitutions to convert the codon of Gln^{26} into a codon coding for Asn. Primer 6 is the sense sequence of *bcl-x* cDNA and contains the nucleotide substitutions to convert the codon of Gln^{26} into a codon coding for Asn. The nucelotide sequence of 5'-end half of Primer 6 is complementary to that of 5'-end half of Primer 5. Two PCR products amplified by PCR were purified by polyacrylamide gel electrophoresis, and two DNA fragments (6 ng each) were mixed to synthesize the respective complementary strand using AmpliTaqGOLD. The condition for synthesis was the same as the above reaction condition 2. After the reaction, a PCR reaction solution (75μl) containing Primer 1, Primer 4 (final concentration: 1 μM each) and AmpliTaqGOLD (2.5U) were added, and the PCR was carried out according to the above reaction condition 1. The 650-bp PCR product was purified by polyacrylamide gel electrophoresis and then treated with the restriction enzyme *Bam*H I. On the other hand, pEF1-BOSbcl-x was treated with *Bam*H I to yield two DNA fragments (5650 bp and 650 bp). The longer DNA fragment (5650 bp) was ligated to the above PCR product in a right orientation to yield the clone pEF1-BOS*bcl-x*Q26N having an amino substitution of Gln26Asn.

Then, two PCR reactions were carried out independently using pEF1-BOS*bcl-x*Q26N as a template. One PCR contained a pair of the above Primer

1 and Primer 7 (SEQ ID NO: 10) and another PCR contained a pair of the above Primer 4 and Primer 8 (SEQ ID NO: 11). The components of the reaction solution (100 μl) were the same as above, and the reactions were performed according to the above-described condition 1. Primer 7 is the antisense sequence of *bcl-x* cDNA and contains the nucleotide substitution to convert the codon of Tyr^{22} into the codon coding for Phe. Primer 8 is the sense sequence of *bcl-x* cDNA and contains the nucleotide substitution to convert the codon of Tyr^{22} into the codon coding for Phe. The nucleotide sequence of 5'-end half of Primer 8 is complementary to that of 5'-end half of Primer 7. Two PCR products amplified by PCR were purified by polyacrylamide gel electrophoresis, and two DNA fragments (6 ng each) were mixed to synthesize the respective complementary strand using AmpliTaqGOLD. The condition for synthesis was the same as the above reaction condition 2. After the reaction, a PCR reaction solution (75 μl) containing Primer 1, Primer 4 (final concentration: 1 μM each) and AmpliTaqGOLD (2.5U) were subjected to PCR according to the above reaction condition 1. The 650-bp PCR product was purified by polyacrylamide gel electrophoresis and then treated with the restriction enzyme *Bam*H I. On the other hand, pEF1-BOS*bcl-x* was treated with *Bam*H I to yield two DNA fragments (5650 bp and 650 bp). The longer DNA fragment (5650 bp) was ligated to the above PCR product in a right orientation to yield the clone pEF1-BOS*bcl-x*Y22F/Q26N having two amino acid substitutions of Tyr22Phe and Gln26Asn.

Finally, pEF1-BOS*bcl-x*R165K and pEF1-BOS*bcl-x*Y22F/Q26N were respectively cleaved with restriction enzymes (*Bgl* II and *Kpn* I). Then, the 1000-bp *BgI* II/*Kpn* I DNA fragment having the amino acid substitutions of Tyr22Phe and Gln26Asn, derived from pEF1-BOS*bcl-x*Y22F/Q26N, was ligated to the 5300-bp *BgI* II/*Kpn* I DNA fragment having the amino acid substitution of Arg165Lys, derived from pEF1-BOS*bcl-x*R165K, to yield the engineered cDNA recombinant vector pEF1-BOS*bcl-x*Y22F/Q26N/R165K coding for the improved protein Bcl-xFNK.

Example 2

Preparation of Transfected Cells

A murine premyeloid cell line FDC-P1 were cultured on RPMI1640 medium containing fetal bovine serum (10%) and a cytokine IL-3 (the supernatant of WEHI cell culture broth). A human leukemia cell line Jurkat were cultured on RPMI1640 medium containing fetal bovine serum (10%). The cells were incubated in a CO_2 incubator (5% CO_2/95% air, 37°C).

The recombinant vector pEF1-BOS*bcl-x*Y22F/Q26N/R165K prepared in Example 1 was amplified in *Escherichia coli* DH5αMCR (GIBCO BRL) and prepared using the Qiagen Plasmid midi Kit (Qiagen). The recombinant vector was cleaved with *Sca* I (one cleavage site) and the resulting linear DNA was dissolved in 1 mM EDTA solution.

The cells (FDC-P1 or Jurkat) were washed 3 times with an ice-cold K-PBS solution (30.8 mM NaCl, 120.7 mM KCl, 8.1 mM Na_2HPO_4, 1.46mM KH_2PO_4) and suspended in K-PBS containig 5 mM $MgCl_2$ (Mg-K-PBS) at 10^7 cells/ml. The cell suspension (0.4 ml) was mixed with Mg-K-PBS solution (0.4 ml) in an ice-cold cuvette (Electroporation Cuvettes Plus, 4-mm Gap, BTX, A Division of Genetronics). Then, the linearized pEF1-BOS*bcl-x*Y22F/Q26N/R165K (10 μg) and the linearized DNA pST-neoB (0.5 μg) containing a drug geneticin-resistant gene were added thereto. Change of the volume by addition of the DNA was kept up to 1%. After 10-min incubation on ice, electroporation was carried out to introduce the recombinant vector into cells using the Gene Pulser (250 μF and 330V, BioRad). After 10-min incubation on ice, the cells were gently suspended in 39 ml of the fresh culture medium in a 100-mm dish and incubated in a CO_2 incubator. After a lapse of 1 day, the cells were divided and placed in a 96-well plate. Geneticin (GIBCO BRL) was added at 200 μg/ml for FDC-P1 cells and at 1 mg/ml for Jurkat cells to select the geneticin-resistant cells.

Example 3

Analysis of expression level of the improved Bcl-xFNK

The expression level of the improved protein Bcl-xFNK in the transfected cells prepared in Example 2 was examined by Western blotting. The cells were washed once with PBS (pH 7.4; NaCl 137 mM, Na_2HPO_4 8.1 mM, KCl 2.68 mM, KH_2PO_4 1.47 mM). After addition of 2% SDS (sodium dodecylsulfate) solution, the cells were disrupted by sonication to solubilize the whole proteins. The proteins were quantitatively analyzed by the BCA Protein Assay (PIERCE), and 20 μg of protein was fractionated on SDS-polyacrylamide gel electrophoresis (Lemli's system). After the electrophoresis, the protein was blotted on a PVDF membrane (Amersham Pharmacia Biotec). The membrane was immersed in a blocking solution containing fetal bovine serum (10%), and then immersed in a TBS solution (Tris-HCl pH 7.4 20 mM, NaCl 136 mM, Tween 80 0.2%) containing the murine monoclonal antibody 105-1 (0.5 μg/ml) that reacts with the C-terminal of rat Bcl-x_L. After incubation at 37°C for 1 hour, the membrane was washed well with TBS, then immersed in a TBS solution containing an HRP (horse radish peroxidase)-binding or AP (alkaline phosphatase)-conjugated secondary antibody, and incubated at 37°C for 1 hour. The HRP-conjugated secondary antibodies binding to Bcl-x_L and Bcl-xFNK were visualized on an X-ray film using the RENAISSANCE kit (NEN Life Science Product). The AP-conjugated antibodies binding to Bcl-x_L and Bcl-xFNK were visualized with the fluoro-image analyzer FLA-2000 (Fuji film) using the ATTOPHOS kit (Boehringer).

The results are shown in Fig. 2. It was confirmed that the cell transfected with the recombinant vector pEF1-BOS*bcl-x*FNK expresses a protein having the same molecular weight (about 30 kDa) as that expressed in the cells transfectd with the clone pEF1-BOS*bcl-x* of the wild-type Bcl-x_L.

Example 4

Confirmation of resistance to the death of the Jurkat*bcl-x*FNK transfectant

For the transfected Jurkat*bcl-x*FNK cells prepared in Example 2, the resistance (non-sensitivity) to a variety of apoptosis-inducing stimuli was examined. The results are shown in Figs. 3 to 13. In these figures, the empty circle (O) represents the transfectant Jurkat*bcl-x*FNK expressing the improved Bcl-xFNK. The filled circle (●) represents the transfectant Jurkat*bcl-x* expressing the wild-type Bcl-xL at the same expression level. The empty square (□) represents Jurkat*vec* cell transfected with the empty vector plasmid DNA. The empty diamond (◇) represents the parent cell Jurkat used in the transfection experiments.

(a) Resistance to apoptosis induced by serum withdrawal

The cells were washed 3 times with PBS and suspended in RPMI1640 medium containing no serum at 1×10^5 cells/ml. The cells were incubated in a CO_2 incubator and the surviving cells were counted by the trypan blue exclusion everyday. The number of the cells was carefully controlled to be less than 5×10^5 cells/ml. When the cell number was expected to exceed the limitation, the culture medium was diluted 2 fold. Every 3 days, the half of the serum-free medium was replaced with the fresh medium.

As shown in Fig. 3, the transfected cells expressing the wild-type Bcl-x_L was resistant to the serum withdrawal and survived longer than the control parent cells and the vector-transfected cells. The transfected cells expressing the improved Bcl-xFNK survived for a longer period than the wild-type Bcl-x_L-expressing cells, from which an excellent apoptosis inhibition effect of Bcl-xFNK was confirmed. Moreover, it was confirmed that the transfected cells could be cultured on serum-free medium.

(b) Resistance to anti-Fas antibody

The cells were suspended in RPMI1640 medium at 1×10^5 cells/ml, to

which anti-Fas antibody (clone CH-11; MBL) was then added at a concentration of 1, 10, 100, or 1000 ng/ml. After incubation for 1 day, the surviving cells were counted by the trypan blue exclusion.

The results are shown in Fig. 4, wherein the number of the cells incubated without the antibody was taken as 100%. As seen clearly from Fig. 4, the transfectant expressing the improved Bcl-xFNK exhibited high resistance to the highly concentrated anti-Fas antibody.

(c) Resistance to a variety of cytotoxic drugs including anti-cancer agents

The cells were suspended in RPMI1640 medium at 1×10^5 cells/ml, to which staurosporine (20 nM), TN-16 (10 μM), camptothecin (10 μM), hydroxyurea (1 mM), trichostatin A (0.25 μg/ml), hydrogen peroxide (0.05 mM), or paraquat (1 mM) was then added. And the cells were incubated. The surviving cells were counted by the trypan blue exclusion everyday.

As seen from the results shown in Figs. 5 to 11, the transfectant expressing the improved Bcl-xFNK exhibited high resistance to all of the cytotoxic drugs tested.

(d) Resistance to heat treatment

The cells were suspended in RPMI1640 medium at 1×10^5 cells/ml, and incubated at 45°C for 10 minutes. The cells were harvest by centrifugation, then suspended in an equal amount of the fresh culture medium, and incubated at 37°C. The surviving cells were counted by the trypan blue exclusion everyday and shown in Fig. 12. In addition, dehydrogenase activities of the cells (100 μl of the culture broth) were determined at 1st day using the Cell Counting Kit (Dojin Chemical) and WST-1 as a substrate (WST-1 Assay). The results are shown in Fig. 13, wherein the enzyme activity of the cells without heat treatment was taken as 100%.

As seen from the above results, it was confirmed that the transfectant expressing the improved Bcl-xFNK exhibited high resistance to heat treatment, and the dehydrogense activity was maintained at high level even after heat treatment.

Example 5

Confirmation of the resistance to the cell death of the FDC-P1*bcl-x*FNK transfectants

For the transfectant FDC-P1*bcl-x*FNK cells prepared in Example 2, the resistance was examined to a variety of apoptosis-inducing stimuli. The results are shown in Figs. 14 to 16. In these figures, the empty marks, ◇, □, △, ▽, and ○ represent 5 independent transfectants, FDC-P1*bcl-x*FNK -1, -2, -3, -4, and -5, respectively. The mark ● represents the transfectant FDC-P1*bcl-x* expressing the wild-type Bcl-x_L at the same expression level. The mark ■ represents FDC-P1*vec* in which the empty vector plasmid DNA has been introduced.

(a) Resistance to TN-16 and staurosporine

The cells were suspended in culture medium at 2×10^5 cells/ml, to which TN-16 (50 μM) and staurosporine (10 nM) was then added. The dehydrogenase activity of the cells (100 μl of the culture broth) was determined daily using the Cell Counting Kit (Dojin Chemical) and WST-1 as a substrate (WST-1 Assay). The enzyme activity was taken as 100% immediately before addition of the agents.

The results are shown in Figs. 14 and 15. It was confirmed that all independent transfectants expressing the improved Bcl-xFNK exhibited high resistance to treatment with TN-16 and staurosporine, and the dehydrogense activity was maintained at high level.

(b) Resistance to apoptosis induced by depletion of the cytokine IL-3

The cells were washed 3 times with PBS and suspended in culture medium containing no IL-3 (but containing serum) at about 5×10^4 cells/ml, and the surviving cells were counted by the trypan blue exclusion everyday. The result are shown in Fig. 16, in which the number of the surviving cells immediately after depletion of IL-3 was taken as 100%. In this experiment, the cells other than

FDC-P1*vec* were diluted 5 times with the fresh medium containing no IL-3 every 3rd day.

As seen clearly from Fig. 16, it was confirmed that the transfectants expressing the improved Bcl-xFNK exhibited higher resistance than the transfectant expressing the wild-type Bcl-x_L to apoptosis induced by depletion of IL-3, and that they could grow even in the absence of IL-3.

Example 6

Preparation of CHO transfectants

Chinese hamster ovary cell CHO were transfected with the recombinant vector pEF1-BOS*bcl-x*Y22F/Q26N/R165K prepared in Example 1.

The CHO cells (1×10^5 cells) were suspended in culture medium DMEM/F-12 (GIBCO BRL) containing 10% fetal bovine serum, and incubated in a 60-mm dish overnight. The linearized pEF1-BOS*bcl-x*Y22F/Q26N/R165K (10 μg) and the linearized pST-neoB (0.5 μg) having a drug Geneticin-resistant gene were introduced into the CHO cells using a SuperFect Transfection Reagent kit (Qiagen). As a control, the linearized empty vector pEF1-BOS or the linearized pEF1-BOSbcl-x were introduced together with the linearized pST-neoB into the CHO cells. After the transfection treatment, the cells were incubated in culture medium DMEM/F-12 containing 10% fetal bovine serum overnight. Geneticin (700 μg/ml) was added, and the incubation was continued to yield the transfected cells. In the same manner as in Example 3, the transfectants expressing the improved protein Bcl-xFNK or the wild-type Bcl-x_L abundantly and to the same level were selected. Thus, CHO*bcl-x*, CHO*bcl-x*FNK, and CHO*vec* (transfected with the empty vector) were obtained.

Example 7

Incubation of the CHO transfectants in serum-free medium

Three transfectants, CHO*bcl-x*, CHO*bcl-x*FNK, and CHO*vec*, prepared in Example 6 were incubated in culture medium DMEM/F-12 containing 10% fetal bovine serum. The cells (1×10^3 cells) were plated in a 100-mm dish containing the medium DMEM/F-12 containing 3% fetal bovine serum. For 5 consecutive days, two thirds of the culture medium was replaced with the DMEM/F-12 containing no fetal bovine serum. The cells were incubated on the medium lacking serum from day 6. The incubation was further continued for another 6 days.

The results are shown in the photograph of Fig. 17. CHO*bcl-x*FNK expressing the improved Bcl-xFNK (Fig. 17C) grew far better than CHO*vec* (Fig. 17B) to which the empty vector was introduced. Additionally, fewer cells were dying or dead and the cells more firmly contacted each other without space in CHO*bcl-x*FNK colonies than those in the colonies of CHO*bcl-x* cells expressing Bcl-x_L (Fig. 17A).

From the above results, it was confirmed that the transfected cells of the invention could grow well in a normal state even in a serum-free medium.

Example 8

Construction of a recombinant vector expressing the TAT-Bcl-xFNK protein

The engineered cDNA coding for Bcl-xFNK prepared in Example 1 was fused to the cDNA coding for the protein transduction domain of TAT protein of HIV virus by 2-step PCR. PCR was carried out using Primer 9 (SEQ ID NO: 14) as the 5'-end primer, Primer 10 (SEQ ID NO: 15) as the 3'-end primer and the recombinant vector pEF1-BOS*bcl-x*Y22F/Q26N/R165K having Bcl-xFNK cDNA as a template. Primer 9 is the sense sequence consisting of the 3'-end of TAT-PTD cDNA and the 5'-end (containing the initiation codon) of Bcl-xFNK cDNA. Primer 10 is the antisense sequence consiting of the 3'-end (containing an termination

codon) of Bcl-xFNK cDNA and the cleavage site for the restriction enzyme *Hind* III. Details of the PCR reaction are as follows

· Reaction solution (volume 100 ml): 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM each dATP, dCTP, dTTP, dGTP, , AmpliTaqGOLD: 2.5U

· Primers: a comibination of Primer 9 and Primer 10 (each primer: 1 μM)

· Template DNA: 50 ng

· Reaction condition 3: 94°C/10 min (94°C/30 sec; 49°C/30 sec; 72°C/1 min) ×15 cycles

After the reaction, the amplified DNA fragment was purified by polyacrylamide gel electrophoresis. To the above-described PCR reaction solution (25 μl) was added the purified DNA fragment (25 ng) and Primer 11 (SEQ ID NO: 16) to synthesize the complementary strand using AmpliTaqGOLD. Primer 16 is the 5'-end sense sequence coding for the amino acid sequence of TAT-PTD flanked by Met (initiation codon)-Gly at the 5'-end and Gly- the initiation codon of Bcl-xFNK cDNA at the 3'-end, as shown in SEQ ID NO: 12. The condition for synthesis is as follows.

· Reaction condition 4: 94°C/10 min (94°C/30 sec; 53°C to 59°C/30 sec; 72°C/1 min) × 5 cycles

After the reaction, the PCR solution (75 ml) containing Primer 12 (SEQ ID NO: 17), Primer 10 (final concentration: 1 μM each), and AmpliTaqGOLD (2.5 U) was added, and the PCR was carried out according to the above reaction condition 3. Primer 12 is a sense sequence coding for Met-Gly and the subsequent N-terminal three amino acid residues of TAT-PTD with a cleavage site of the restriction enzyme *Nde* I at the 5'-end. The amplified DNA fragment was purified by polyacrylamide gel electrophoresis. After cleavage with *Nde* I, the cleaved end was made blunt with T4DNA polymerase and further subjected to be digested with *Hind* III. The *Escherichia coli* expression vector pROEX1 (Life Technologies) was cleaved with *Nco* I, then made blunt with nuclease S1, followed by digestion with Hind III. Two DNAs were ligated each other to yield the recombinant vector

pROEX1- -*bcl-x*Y22F/Q26N/R165K coding for TAT-Bcl-xFNK in which a protein-transduction-domain peptide of TAT protein is fused at the N-terminal.

Example 9

Preparation of TAT-Bcl-xFNK protein

TAT-Bcl-xFNK protein was expressed in *Escherichia coli* and partially purified as described below. *Escherichia coli* DH5αMCR carrying pROEX1-*bcl-x*Y22F/Q26N/R165K was incubated on 1000 ml of LB liquid medium (5 g yeast extract, 10 g Bactotrypton, and 5 g NaCl) containing ampicillin (50 μg/mg) with shaking at 37°C. When the cells reached at the logarithmic growth phase (O.D.600= 0.5), IPTG (isopropyl-1-thio-β-galactoside; final concentration 1 mM) was added, and the incubation was continued for 2 hours. TAT-Bcl-xFNK protein was prepared from a soluble fraction and an insoluble fraction (inclusion body) after disrupting the cells. The protein was prepared from the soluble fraction as follows. The harvested cells were washed 3 times with PBS, then suspended in 40 ml of Buffer A (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF), and disrupted by ultrasonication. After centrifugation, TAT-Bcl-xFNK was purified from the supernatant by antibody-affinity chromatography using a column in which was packed a monoclonal antibody 35-32 bound to carriers, said monoclonal antibody being derived from a mouse and recognizing the N-terminal region of rat Bcl-x_L. TAT-Bcl-xFNK was bound to the antibody, washed, and then eluted with an eluate (50 mM Glycine-HCl pH 2.7, 50 mM NaCl). The eluate was neutralized with 2M Tris-HCl (pH 9.0) and concentrated by Centricon (Amicon). Dialysis against PBS gave TAT-Bcl-xFNK preparation for use in the following experiment. TAT-Bcl-xFNK was prepared from the insoluble fraction (inclusion body) as follows. The harvested cells were washed 3 times with PBS, then suspended in 36 ml of Buffer A (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT) containing DTT in place of PMSF,

and disrupted by ultracentrifugation. Triton X-100 (final concentration 1%) was added, and the mixture was placed on ice for 10 minutes. The inclusion body containing TAT-Bcl-xFNK was precipitated by centrifugation, and washed twice with Buffer A containing Triton X-100. Finally, the inclusion body was solubilized in PBS containing 7M urea and 1 mM DTT. This preparation was confirmed to contain TAT-Bcl-xFNK protein of 70% purity by SDS-polyacrylamide gel electrophoresis and used in the following experiment.

Example 10

Incorporation of TAT-Bcl-xFNK protein in cells

TAT-Bcl-xFNK protein (1 μM) was added to DMEM/F-12 (Life Technologies) containing 10% FBS (fetal bovine serum), a medium for HeLa cells cultured in a Slide Chamber (Lab-Tek), and the cells were incubated in a CO_2 incubator for 24 hours. The cells were then washed twice with PBS. The cells were fixed with paraformaldehyde (4%) dissolved in PBS at room temperature for 45 min. The cells were washed 3 times with PBS (5 minutes/round), and incubated in PBS containing 10% FBS for 20 min. The cells were washed 3 times with PBS (5 minutes/round), and treated with 1.5% FBS-PBS solution containing an anti-rat Bcl-x_L monoclonal antibody 35-32 (murine origin) for 30 min. The cells were washed 3 times with PBS (5 minutes/round), and treated with 1.5% FBS-PBS solution containing an anti-mouse IgG antibody conjugated with FITC, for 30 minutes. The cells were washed 3 times with PBS (5 minutes/round), mounted in PBS, and sealed to observe under a fluorescence microscope.

The results are shown in Fig. 18. Spotted fluorescence characteristic to FITC was observed in the cells. This fluorescence signal could not be observed in the cells incubated without TAT-Bcl-xFNK protein. Further, even though the TAT-Bcl-xFNK protein was added, when the cells were not treated with the primary antibody (35-32), no signal was observed. These results indicate that the

TAT-Bcl-xFNK protein added to the culture medium has passed through the cellular membrane and has been incorporated in the cells.

Example 11

Introduction of TAT-Bcl-xFNK into the chondrocyte of cartilage slice culture and confirmation of the cell death-inhibiting activity

Cartilage was obtained from the femoral bone head of Osteoarthritis patients undergoing total hip arthroplasty. The cartilage tissue above the subchondral bone (10×10 mm; 1.2 mm in thickness) was aseptically sliced using a single-edged razor. The cartilage slice was placed in a 24-well plate and incubated with an DMEM/Ham F-12 mixed medium (Life Technologies) containing 20% FBS (fetal bovine serum) at 37°C in a CO_2 incubator. For a comparative experiment, the expression vector of TAT-Bcl-x_L was constructed in the same manner as TAT-Bcl-xFNK, and TAT-Bcl-x_L protein was partially purified from *Escherichia coli* (TAT-Bcl-x_L preparation has the same purity). TAT-Bcl-xFNK (prepared from the inclusion body) or TAT-Bcl-x_L (prepared from the inclusion body) were added to the culture medium at a concentration of 0.2 μM. As a control, an equal amount of PBS (a solvent used to solubilize the proteins) containing 7M urea and 1 mM DTT was added. The culture medium containing the protein was changed on day 4 and day 7. After incubation for 4 days and 9 days, the cartilage slice was frozen to prepare frozen sections using a cryostat. The sections were stained with hematoxylin-eosin to evaluate the death of chondrocyte. As shown in Figs. 19 to 24, the results indicate that TAT-Bcl-xFNK inhibits the death of chondrocyte more strongly than TAT-Bcl-x_L, and the difference between them is more remarkable on day 9. It was shown that TAT-Bcl-xFNK protein in the culture medium was incorporated into the chondrocytes buried in the cartilage tissue to exhibit its powerful activity to inhibit

cell death.

Example 12

Administration of TAT-Bcl-xFNK to mice and confirmation of the inhibition of the death of hepatocytes caused by steroid hormone

Three 8-week old mice (C56BL, 20 g body weight, female) were divided into 3 groups (A, B and C). To the mouse of Group A was intraperitonealy administered PBS solution (0.8 ml) containing 100 μg of TAT-Bcl-xFNK protein (prepared from the soluble fraction). To the mice of Group B and Group C (control) was intraperitonealy administered PBS (0.8 ml) in the same manner. The mice were put back into cages, and after a lapse of 3 hours 0.5 ml of 25% ethanol/PBS solution containing 0.5 mg of a steroid hormone (dexamethasone) was intraperitonealy administered to the mice of Group A and Group B. To the mouse of Group C was intraperitonealy administered 0.5 ml of 25% ethanol/PBS solution. They were put back into cages, and after a lapse of 3 hours killed. Their livers were taken out, and frozen to prepare frozen sections with a cryostat. The sections were stained with hematoxylin-eosin to evaluate the death of hepatocyte. Degeneration of the hepatic tissue and cell death caused by dexamethasone in Group B as shown in Fig. 26 were markedly inhibited by pre-administration of TAT-Bcl-xFNK (Group A; Fig. 25). The degree was shown to be better than that of the control (Group C; Fig. 27).

The above results indicate that the protein TAT-Bcl-xFNK intraperitoneally administered is delivered into the hepatic cells to strongly inhibit cell death caused by dexamethasone.

Industrial Applicability

As described above in detail, the invention of the present application provides a engineered cDNA producing an improved protein of rat Bcl-x_L which exhibits the more enhanced activity to inhibit cell death, a recombinant vector having the engineered cDNA, and cells transfected with the recombinant vectors. The transfected cells can be proliferated in a serum-free medium, and are useful for, for example, cell culture systems to efficiently produce useful substances such as physiologically active substances, monoclonal antibodies, and the like. Moreover, the invention of the present application provides an improved protein of rat Bcl-xL which exhibits the more enhanced activity to inhibit cell death. The improved protein, when it has a protein-transduction-domain peptide, is incorporated into cells and transiently present in the cells to inhibit apoptosis/cell death. The protein is, accordingly, useful as, for example, an ingredient for remedies for various diseases accompanied with cell death or for additives for stably maintaining trans-planted cells or organs.

CLAIMS

1. A genetically engineered cDNA of the rat *bcl-x* gene, which has at least one substitution selected from the substitutions that change residues 22 Tyr to Phe, residues 26 Gln to Asn and residues 165 Arg to Lys, in the coding region of rat *bcl-x* cDNA of SEQ ID NO: 1.

2. The genetically engineered cDNA of claim 1, which is attached at its 5'-end with an oligonucleotide encoding a protein-transduction-domain peptide.

3. The genetically engineered cDNA of claim 2, wherein the oligonucleotide encodes the amino acid sequence of SEQ ID NO: 12 or 13.

4. A recombinant vector carrying the genetically engineered cDNA of any one of claims 1 to 3.

5. A cell into which the recombinant vector of claim 4 was introduced.

6. An improved protein produced by the genetically engineered cDNA of claim 1, which has at least one amino acid substitution in SEQ ID NO: 2, which amino acid substitution is selected from the substitutions of residues 22 Tyr to Phe, residues 26 Gln to Asn and residues 165 Arg to Lys.

7. The improved protein of claim 6, which is attached at the N-terminal with a protein-transduction-domain peptide.

8. The improved protein of claim 7, wherein the protein-transduction-domain peptide is an oligopeptide having the amino acid sequence of SEQ ID NO: 12 or 13.

ABSTRACT

The present invention provides an genetically engineered cDNA of the rat *bcl-x* gene, which has at least one substitution selected from the substitutions that change residues 22 Tyr to Phe, residues 26 Gln to Asn and residues 165 Arg to Lys, in the coding region of rat *bcl-x* cDNA of SEQ ID NO: 1, a recombinant vector containing the engineered cDNA, a cell into which the recombinant vector was introduced, and an improved protein of Bcl-x_L. The improved protein of Bcl-x_L is useful as an ingredient for remedies for various diseases accompanied with cell death since it effectively inhibit cell death such as apoptosis.

Fig.1

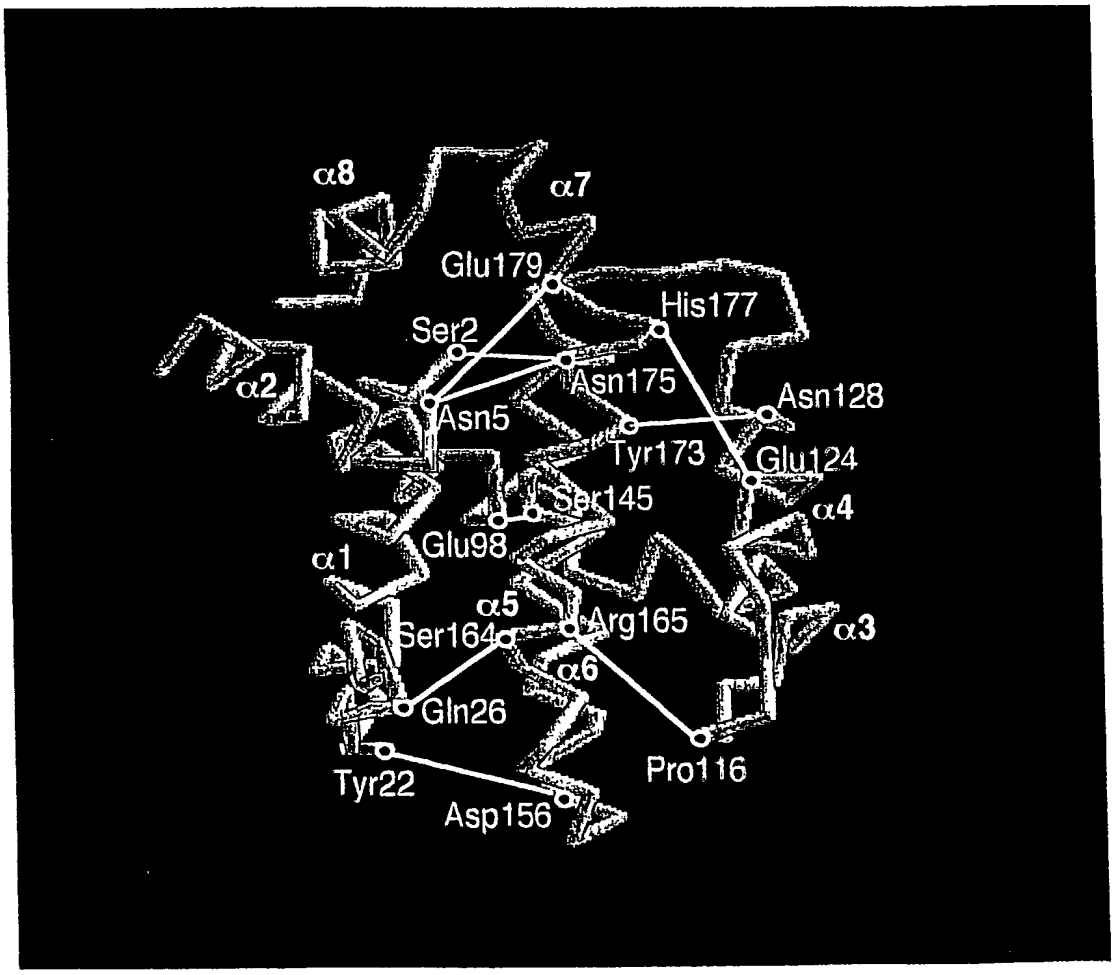

α8
α7
Glu179
His177
Ser2
Asn175
α2
Asn5
Asn128
Tyr173
Glu124
Ser145
α4
Glu98
α1
α5
Arg165
Ser164
α3
α6
Gln26
Pro116
Tyr22
Asp156

Fig.2

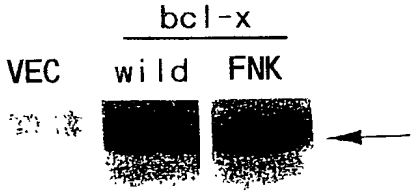

bcl-x
VEC
wild
FNK

Fig.3

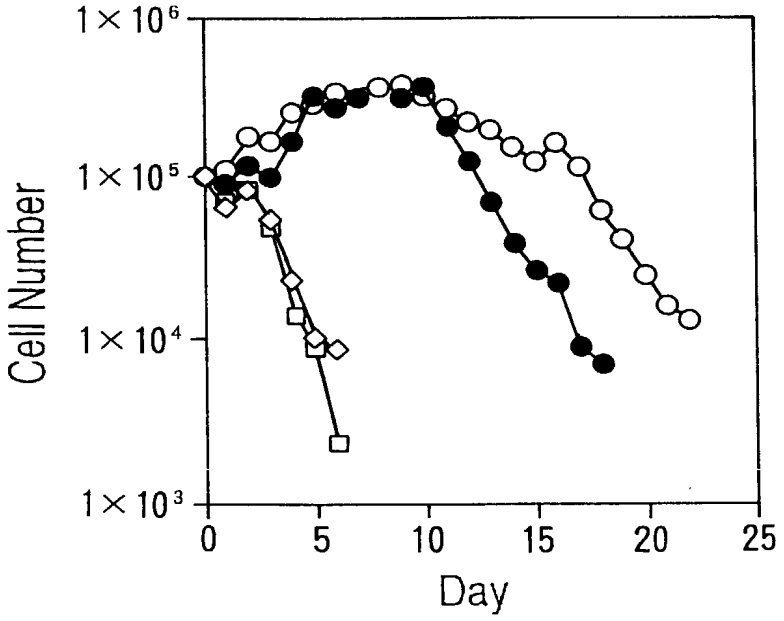

1×10⁶
1×10⁵
1×10⁴
1×10³
Cell Number
0
5
10
15
20
25
Day

Fig.4

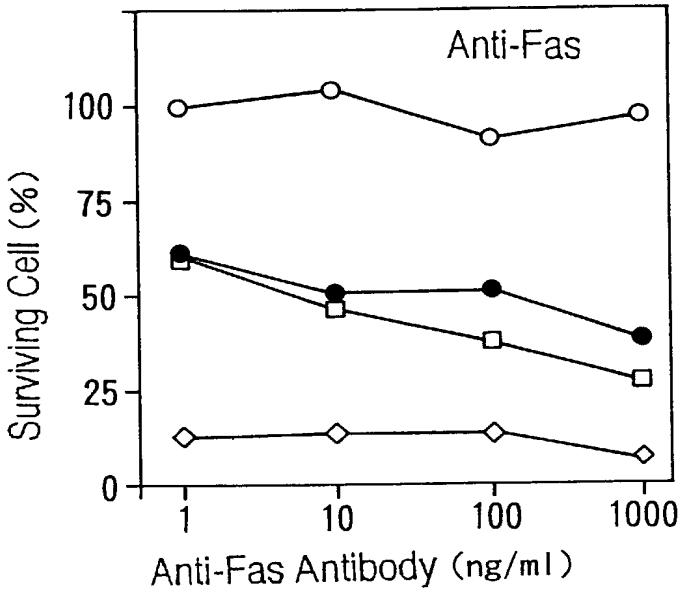

Anti-Fas
100
75
50
25
0
Surviving Cell (%)
1
10
100
1000
Anti-Fas Antibody (ng/ml)

Fig.5

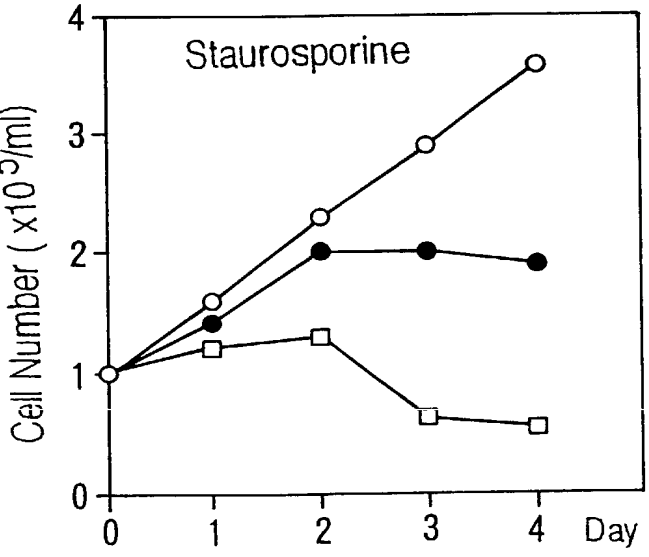

Staurosporine
4
3
2
1
0
Cell Number (x10 5/ml)
0
1
2
3
4
Day

Fig.6

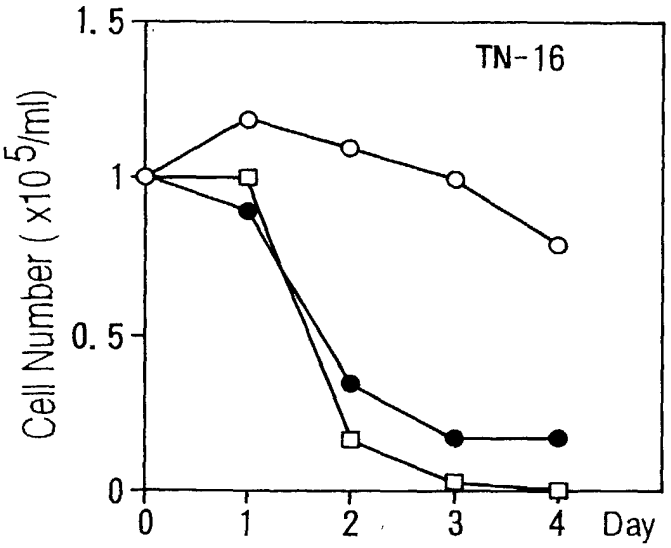

TN-16
Cell Number (x10 5/ml)
1.5
1
0.5
0
0
1
2
3
4
Day

Fig.7

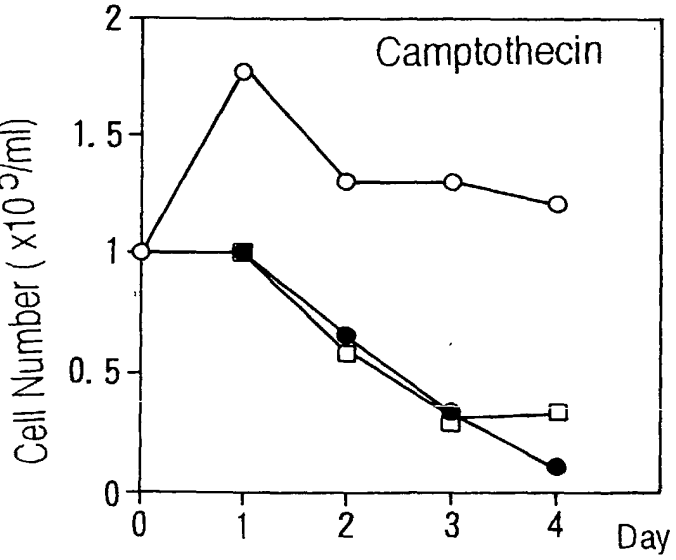

Camptothecin
Cell Number (x10 5/ml)
2
1.5
1
0.5
0
0
1
2
3
4
Day

Fig.8

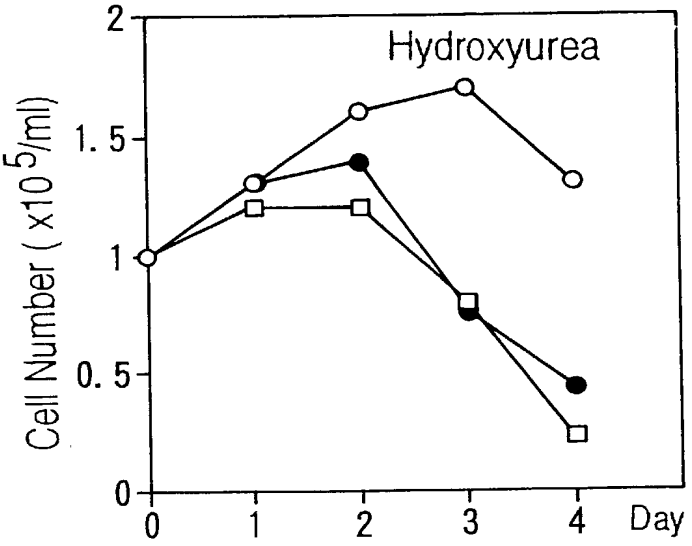

Hydroxyurea
Cell Number (x10 5/ml)
2
1. 5
1
0. 5
0
0
1
2
3
4
Day

Fig.9

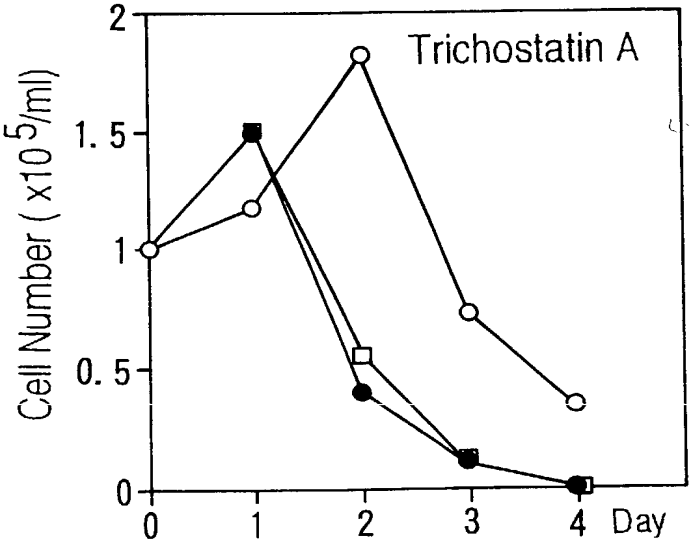

Trichostatin A
Cell Number (x10 5/ml)
2
1. 5
1
0. 5
0
0
1
2
3
4
Day

Fig.10

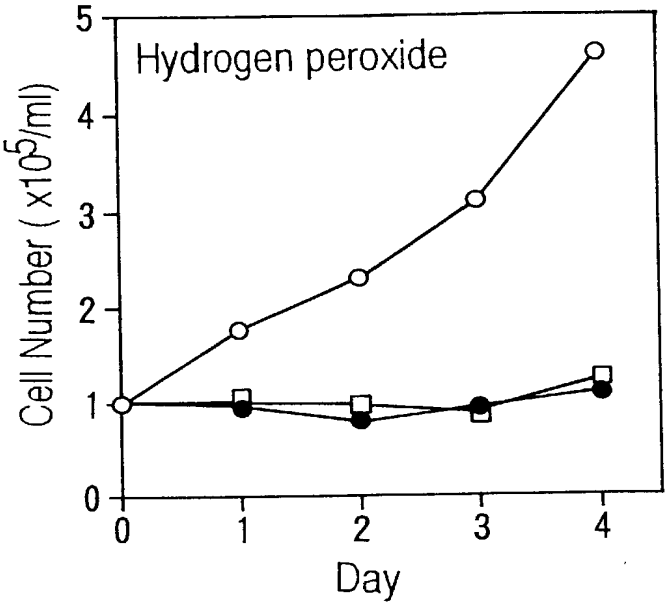

Hydrogen peroxide
Cell Number (x10^5/ml)
0
1
2
3
4
5
Day

Fig.11

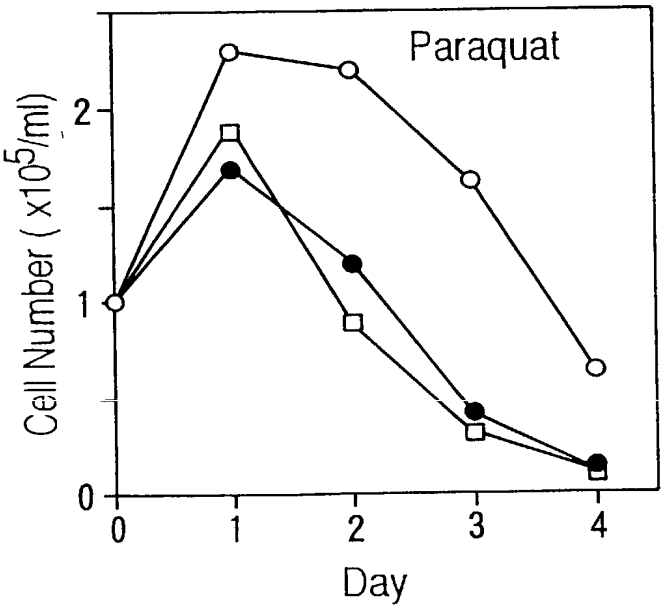

Paraquat
Cell Number (x10^5/ml)
0
1
2
Day

Fig.12

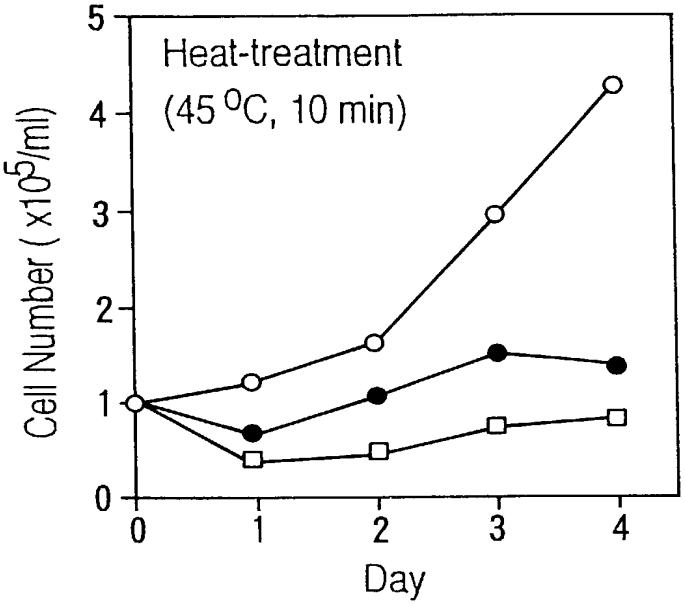

Heat-treatment
(45 °C, 10 min)
Cell Number (x10^5/ml)
0
1
2
3
4
5
0
1
2
3
4
Day

Fig.13

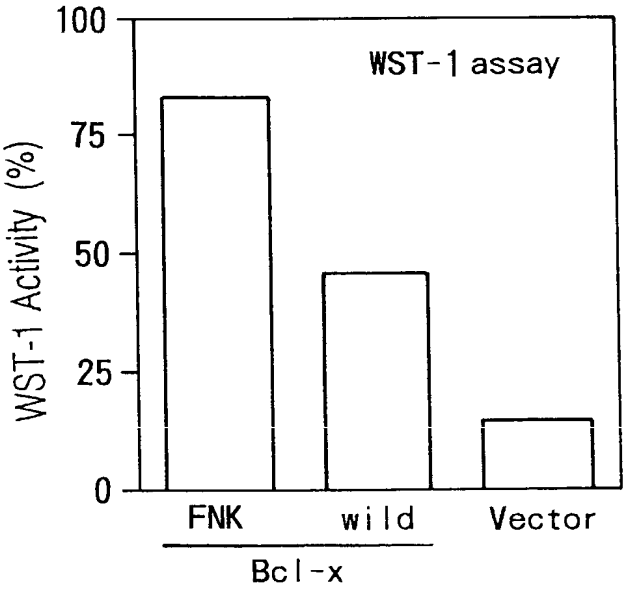

WST-1 assay
WST-1 Activity (%)
0
25
50
75
100
FNK
wild
Vector
Bcl-x

Fig.14

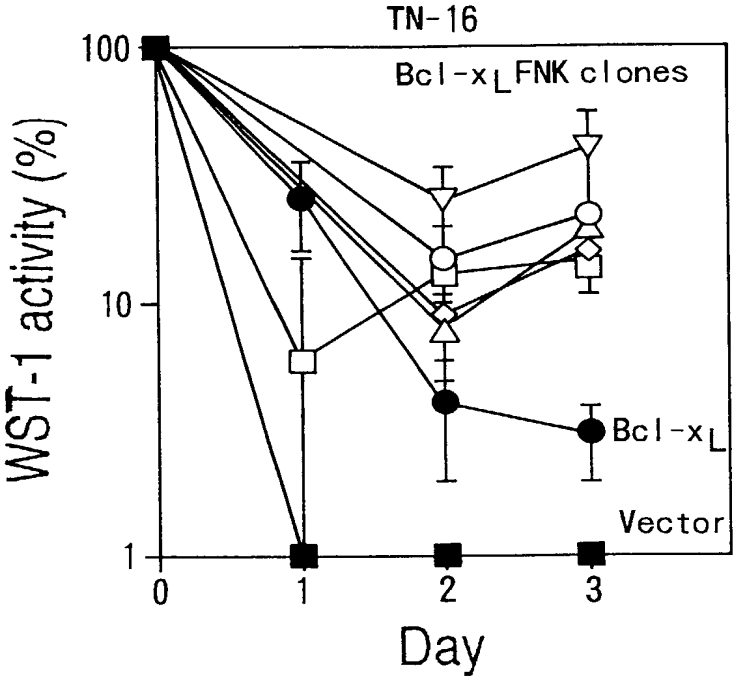

TN-16
Bcl-xL FNK clones
100
10
1
WST-1 activity (%)
Bcl-xL
Vector
0
1
2
3
Day

Fig.15

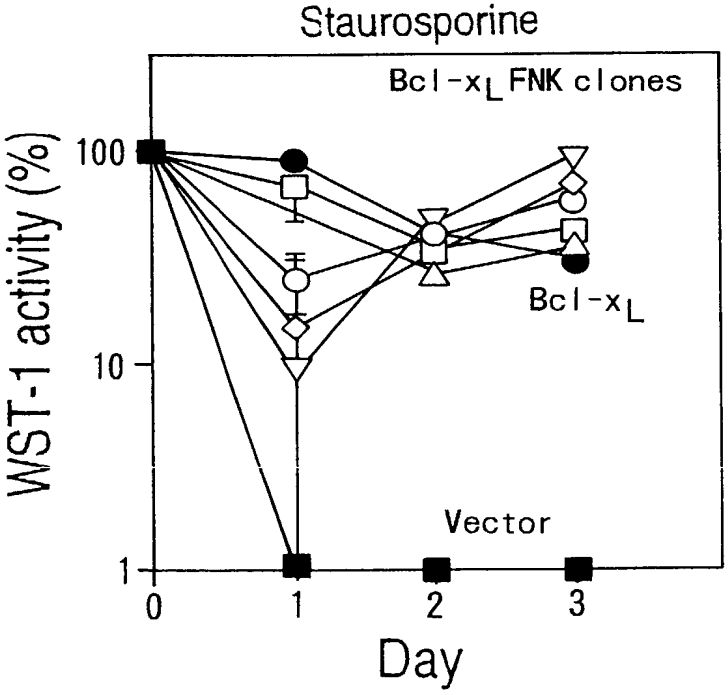

Staurosporine
Bcl-xL FNK clones
100
10
1
WST-1 activity (%)
Bcl-xL
Vector
0
1
2
3
Day

Fig.16

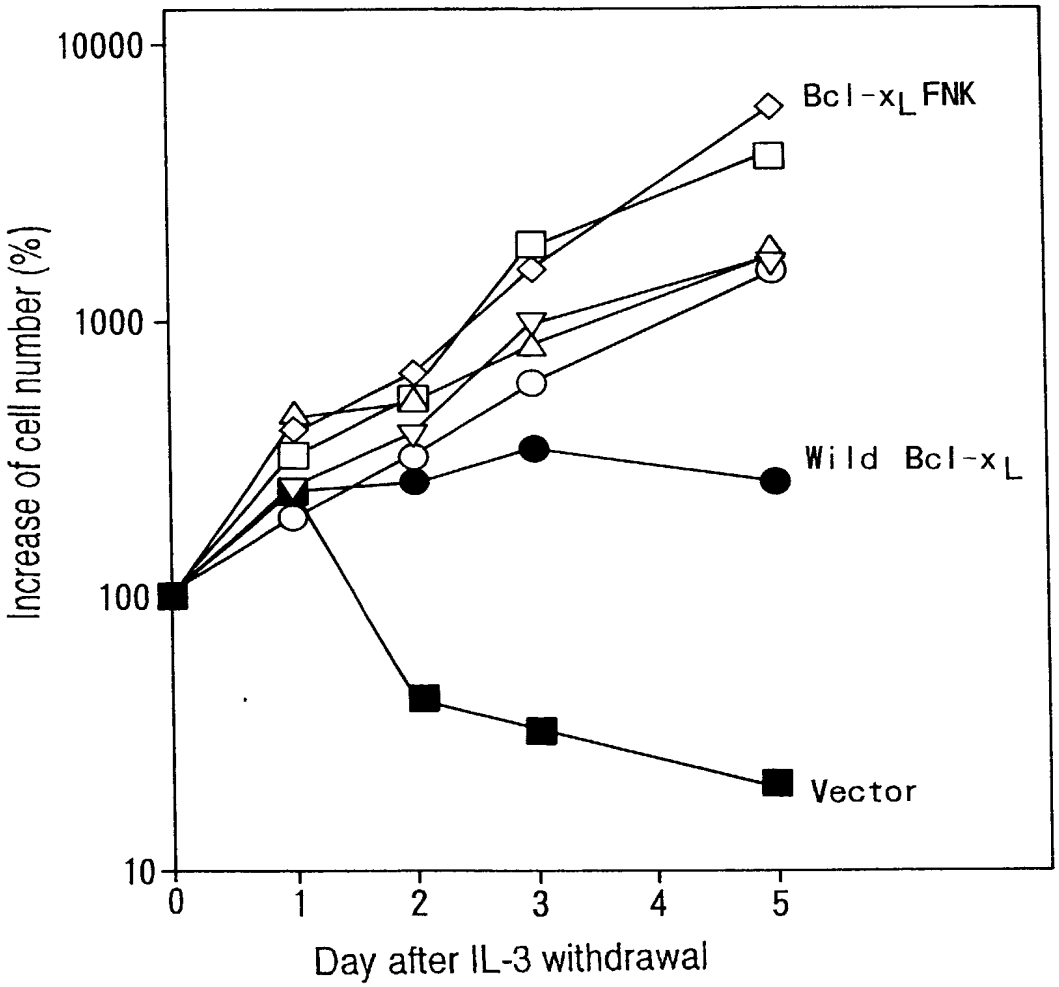
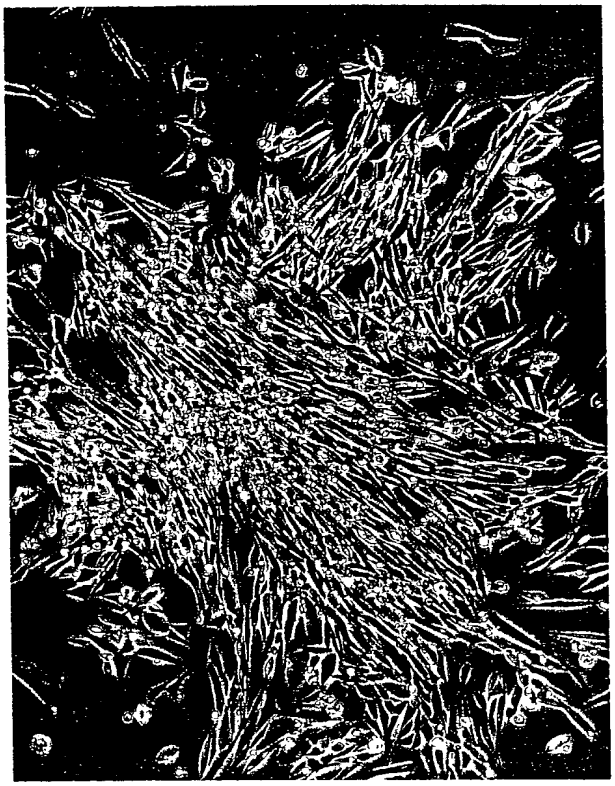
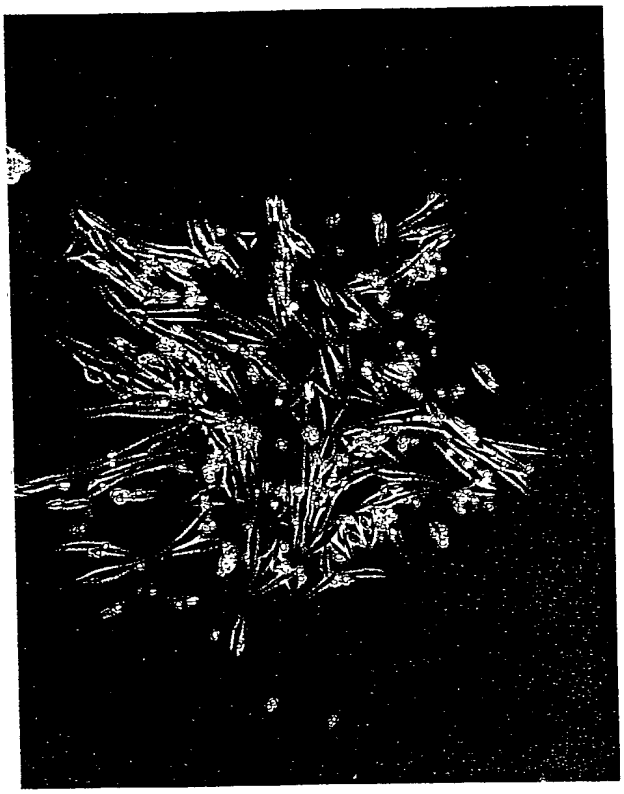

10000
1000
100
10
Increase of cell number (%)
0
1
2
3
4
5
Day after IL-3 withdrawal
Bcl-xL FNK
Wild Bcl-xL
Vector

Fig.17

A



B



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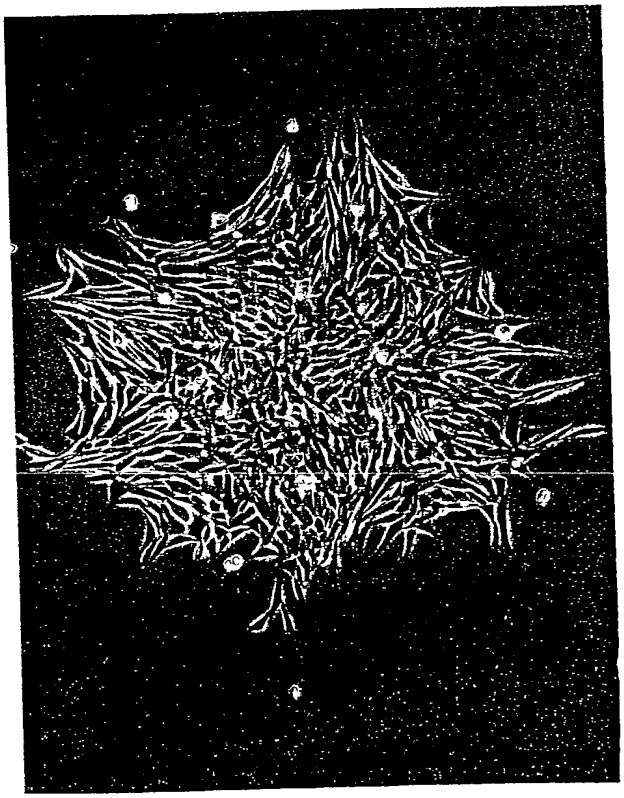

Fig.18

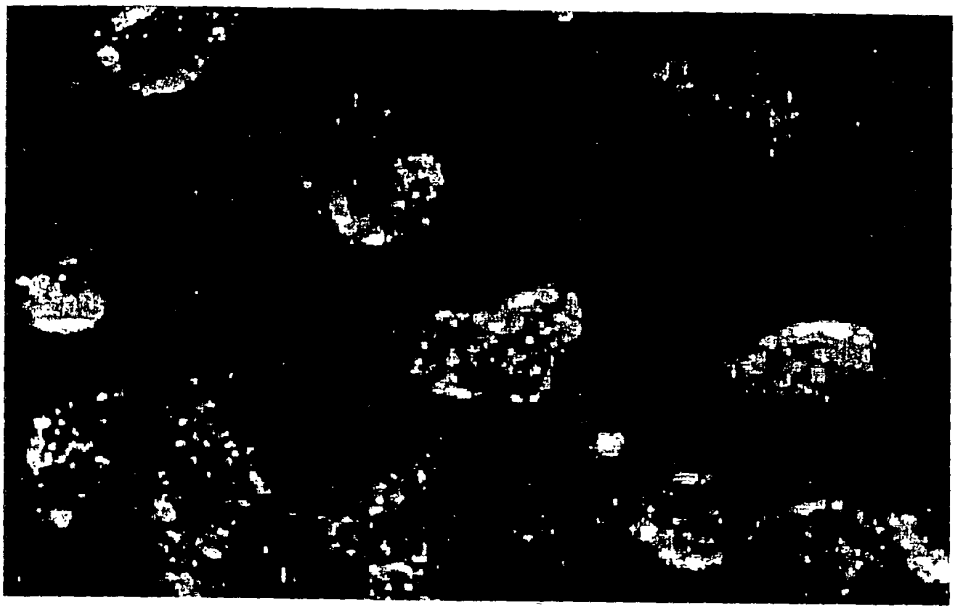

Fig.19

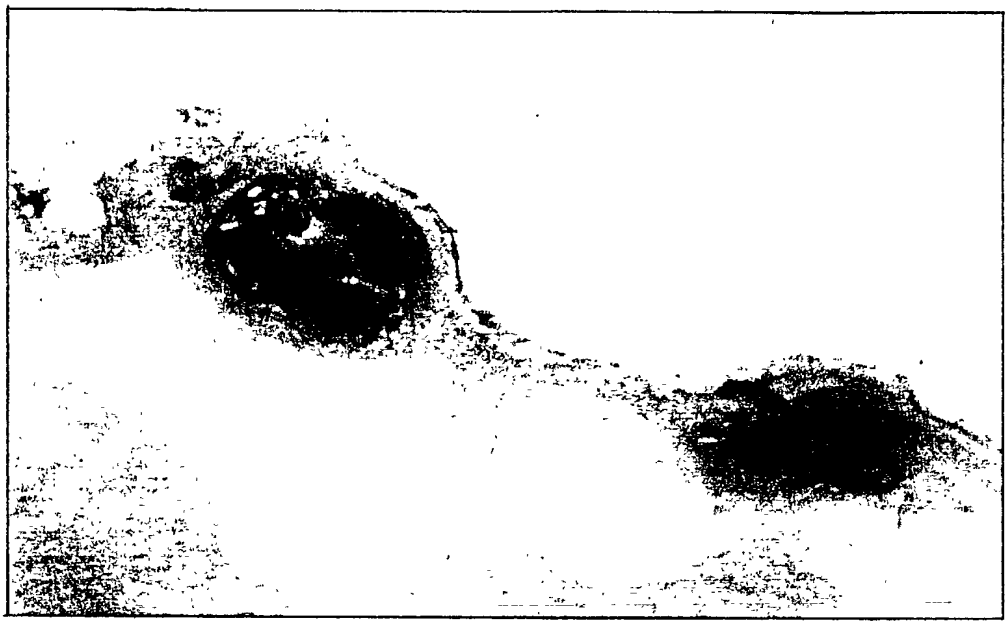

Fig.20

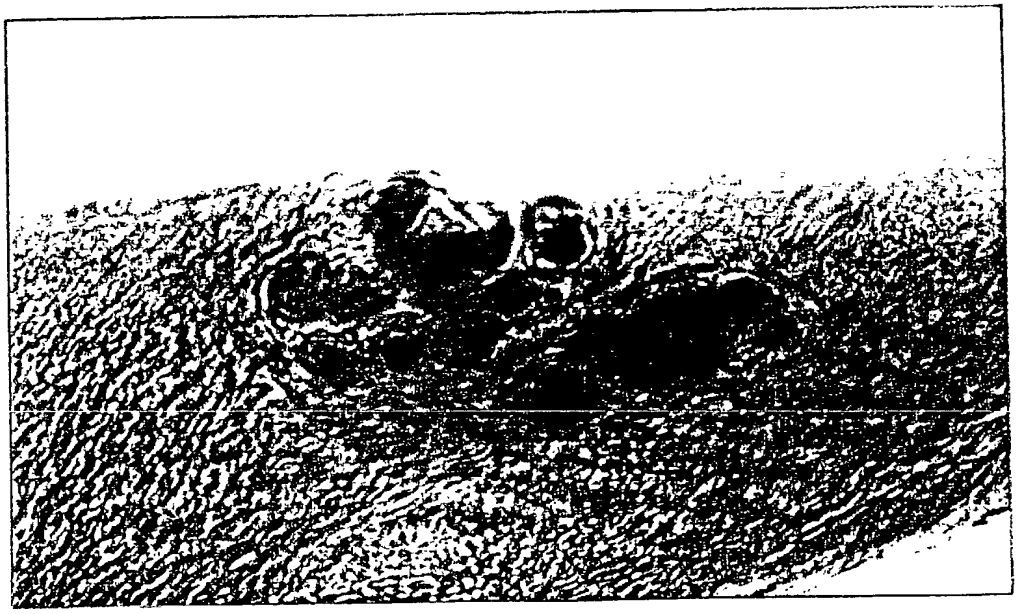

Fig.21

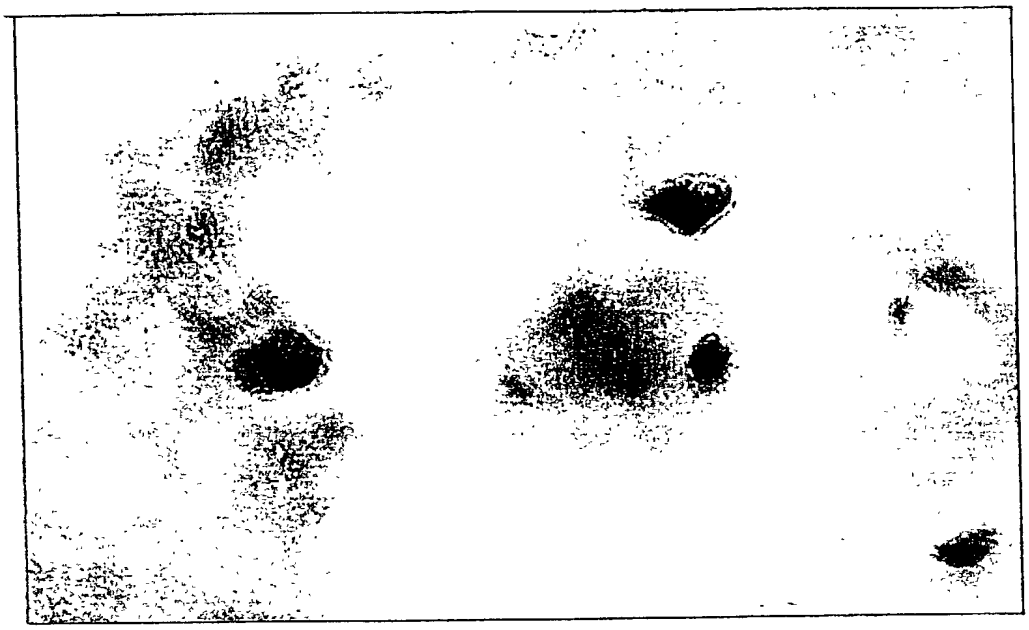

Fig.22

Fig.23

Fig.24

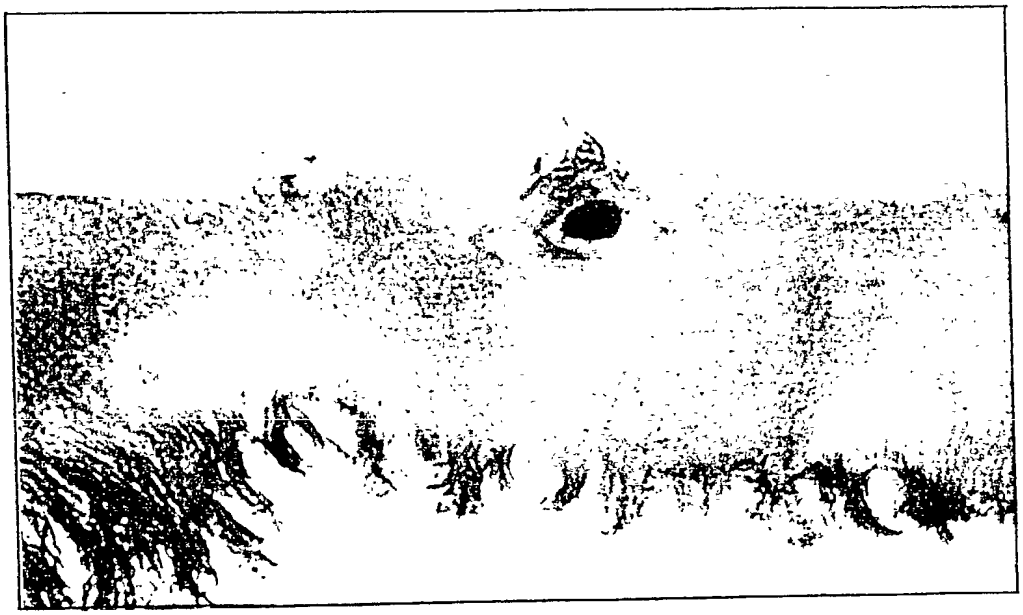

Fig.25

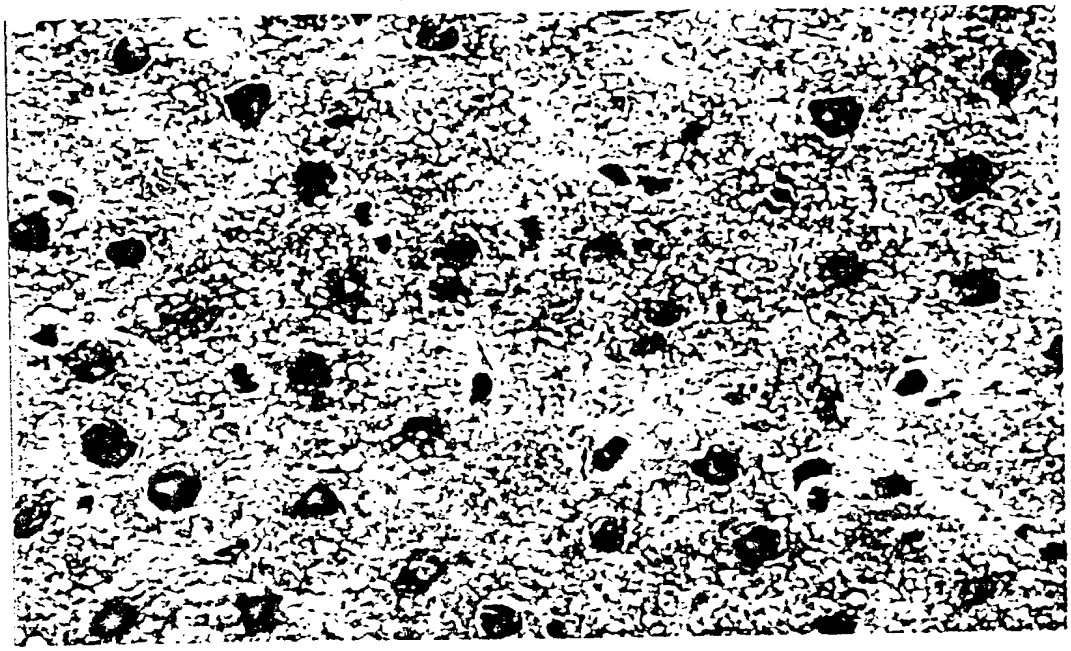

Fig.26

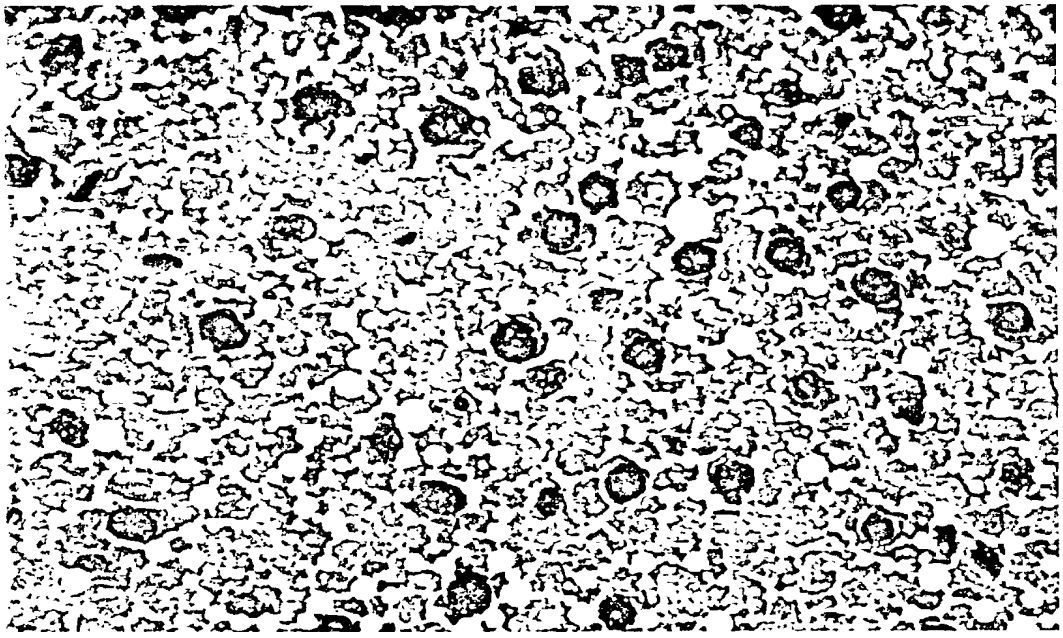

Fig.27

Rev. 3-21-01 Effective March 1998

DECLARATION AND POWER OF ATTORNEY FOR U.S. PATENT APPLICATION

(X) Original () Supplemental () Substitute (X) PCT () DESIGN

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that I verily believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Title: A GENETICALLY ENGINEERED cDNA OF RAT bcl-x GENE AND AN IMPROVED PROTEIN

of which is described and claimed in:
() the attached specification, or
(X) the specification in application Serial No. _____, filed February 19, 2002, and with amendments through _____, or
() the specification in International Application No. PCT/JP00/05502, filed August 17, 2000, and as amended on _____ (if applicable).

I hereby state that I have reviewed and understand the content of the above-identified specification, including the claims, as amended by any amendment(s) referred to above.

I acknowledge my duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim priority benefits under Title 35, United States Code, §119 (and §172 if this application is for a Design) of any application(s) for patent or inventor's certificate listed below and have also identified below any application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

COUNTRY	APPLICATION NO.	DATE OF FILING	PRIORITY CLAIMED
Japan	1999-230642	August 17, 1999	Yes

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	U.S. FILING DATE	STATUS: PATENTED, PENDING, ABANDONED

And I hereby appoint Michael R. Davis, Reg. No. 25,134; Matthew M. Jacob, Reg. No. 25,154; Warren M. Cheek, Jr., Reg. No. 33,367; Nils Pedersen, Reg. No. 33,145; Charles R. Watts, Reg. No. 33,142; and Michael S. Huppert, Reg. No. 40,268, who together constitute the firm of WENDEROTH, LIND & PONACK, L.L.P., as well as any other attorneys and agents associated with Customer No. 000513, to prosecute this application and to transact all business in the U.S. Patent and Trademark Office connected therewith.

I hereby authorize the U.S. attorneys and agents named herein to accept and follow instructions from NISHIZAWA & ASSOCIATES as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and myself. In the event of a change in the persons from whom instructions may be taken, the U.S. attorneys named herein will be so notified by me.

ATTACHMENT B

Direct Correspondence to Customer No:	Direct Telephone Calls to:
000513 PATENT TRADEMARK OFFICE	WENDEROTH, LIND & PONACK, L.L.P. 2033 "K" Street, N.W., Suite 800 Washington, D.C. 20006-1021 Phone (202) 721-8200 Fax (202) 721-8250

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Post Office Address	ADDRESS 1754-35, Hon-machida, Machida-shi, Tokyo, Japan	CITY	STATE OR COUNTRY / ZIP CODE
Full Name of Second Inventor	FAMILY NAME ASOH	FIRST GIVEN NAME Sadamitsu	SECOND GIVEN NAME
Residence & Citizenship	CITY Kanagawa	STATE OR COUNTRY Japan	COUNTRY OF CITIZENSHIP Japan JPX
Post Office Address	ADDRESS 224, Kashimada, Saiwai-ku, Kawasaki-shi, Kanagawa, Japan	CITY	STATE OR COUNTRY / ZIP CODE
Full Name of Third Inventor	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
Residence & Citizenship	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
Post Office Address	ADDRESS	CITY	STATE OR COUNTRY / ZIP CODE
Full Name of Fourth Inventor	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
Residence & Citizenship	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
Post Office Address	ADDRESS	CITY	STATE OR COUNTRY / ZIP CODE
Full Name of Fifth Inventor	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
Residence & Citizenship	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
Post Office Address	ADDRESS	CITY	STATE OR COUNTRY / ZIP CODE
Full Name of Sixth Inventor	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
Residence & Citizenship	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
Post Office Address	ADDRESS	CITY	STATE OR COUNTRY / ZIP CODE

I further declare that all statements made herein of my own knowledge are true, and that all statements on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1st Inventor Shigeo Ohta Date March 20, 2002
Shigeo OHTA

2nd Inventor Sadamitsu Asoh Date March 20, 2002
Sadamitsu ASOH

3rd Inventor _____ Date _____

4th Inventor _____ Date _____

5th Inventor _____ Date _____

6th Inventor _____ Date _____

The above application may be more particularly identified as follows:

U.S. Application Serial No. _____ Filing Date February 19, 2002

Applicant Reference Number 00-F-034US/YS Atty Docket No. 2002_0256A

Title of Invention A GENETICALLY ENGINEERED cDNA OF RAT bcl-x GENE AND AN IMPROVED PROTEIN

SEQUENCE LISTING

<110> Japan Science and Technology Corporation

<120> A mutagenized rat bcl-xL cDNA and a modified protein therefrom

<130> NP00291-YS

<140> PCT/JP00/05502

<141> 2000-08-17

<150> JP11-230642

<151> 1999-08-17

<160> 17

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<222> (72)..(773)

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<302> An additional form of rat Bcl-x, Bcl-xbeta, generated by an unspliced RNA, promotes apoptosis in promyeloid cells.

<303> J. Biol. Chem.

<304> 271

<305> 22

<306> 13258-13265

<307> May 31, 1996

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15 20 25

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Val Leu Val Ser Arg Ile Ala Ser Trp Met Ala Thr Tyr Leu Asn Asp

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His Leu Glu Pro Trp Ile Gln Glu Asn Gly Gly Trp Asp Thr Phe Val

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Ser Ala Ile Asn Gly Asn Pro Ser Trp His Leu Ala Asp Ser Pro Ala

50 55 60

Val Asn Gly Ala Thr Gly His Ser Ser Ser Leu Asp Ala Arg Glu Val

65 70 75 80

Ile Pro Met Ala Ala Val Lys Gln Ala Leu Arg Glu Ala Gly Asp Glu

85 90 95

Phe Glu Leu Arg Tyr Arg Arg Ala Phe Ser Asp Leu Thr Ser Gln Leu

100 105 110

His Ile Thr Pro Gly Thr Ala Tyr Gln Ser Phe Glu Gln Val Val Asn

115 120 125

Glu Leu Phe Arg Asp Gly Val Asn Trp Gly Arg Ile Val Ala Phe Phe

130 135 140

Ser Phe Gly Gly Ala Leu Cys Val Glu Ser Val Asp Lys Glu Met Gln

145 150 155 160

Val Leu Val Ser Lys Ile Ala Ser Trp Met Ala Thr Tyr Leu Asn Asp

165 170 175

His Leu Glu Pro Trp Ile Gln Glu Asn Gly Gly Trp Asp Thr Phe Val

180 185 190

Asp Leu Tyr Gly Asn Asn Ala Ala Ala Glu Ser Arg Lys Gly Gln Glu

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Leu Leu Gly Ser Leu Phe Ser Arg Lys

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United States Patent & Trademark Office

Office of Initial Patent Examination -- Scanning Division

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